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THE UNIVERSITY OF ALBERTA

TRANSCRIPTIONAL REGULATION OF TWO CYTOTOXIC T CELL-SPECIFIC SERINE PROTEASE GENES

by

©

CHANTAL J. FREGEAU

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA
SPRING, 1992

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Dated October 23 1991

"Everything should be made as simple as possible, but not simpler."

_ Albert Einstein (1897-1955)

"If criticism had any real power to harm, the skunk would be extinct by now."

_ Fred Allen (1894-1956)

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "**Transcriptional Regulation of Two Cytotoxic T Cell-Specific Serine Protease Genes**" submitted by Chantal J. Frégeau in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Date: October 21, 1991

À mon père, qui a su éveiller en moi cette fascination pour la vie et cette soif de comprendre. Son éternel détermination et son dévouement au travail ont été de bons modèles et d'excellents chevaux de bataille tout au long des cinq dernières années.

ABSTRACT

Serine proteases are active participants in the process of cell-mediated killing. The molecular basis of the transcriptional regulation of two cytotoxic T lymphocyte (CTL)-specific serine protease genes, referred to as C11 and B10, was investigated. A transfection protocol was first adapted from the standard DEAE dextran method for the introduction of exogenous DNA into the cytotoxic type II MTL2.8.2 cell line. A variety of C11 and B10 5'-deletion/*cat* fusion constructs was then tested in different cell types in a transient expression assay. Both C11 and B10 5'-flanking regions were shown to contain sequences responsible for their CTL-specific expression. However, further analysis revealed that this specificity is achieved through the combined action of many different factors that bind to various target sequences. Distinct modulatory regions located within the DNAse1 hypersensitive sites of C11 and B10 were identified. While some effected an up-regulation of the reporter *cat* gene expression, others had a negative effect on it. The function of these elements always depended on their surrounding context. In addition, some of the regulatory elements identified shared homology with known regulatory sequences while others appeared to be novel motifs.

Transfection experiments performed in the presence of sodium butyrate, an inducer of transcription, provided further evidence for the modular and distinct nature of the C11 and B10 5'-upstream sequences. Both C11-directed exogenous *cat* and C11 endogenous gene expression were affected in a similar way following treatment of MTL2.8.2 cells with butyrate. Likely, B10-driven *cat* and B10 endogenous gene expression showed the same pattern of modulation. Butyrate-sensitive areas were identified in each gene but C11 was particularly responsive to the agent. The locations of these sensitive areas were distinct for C11 and B10 but always mapped within DNAse1 hypersensitive sites where the regulatory regions were defined earlier. In addition, the identified responsive elements shared

homologies with previously described sequences capable of conferring butyrate-responsiveness to heterologous promoters.

Preliminary DNAse1 footprinting analyses indicated that the 5'-ends of C11 and B10 have very different protection profiles. All these experiments suggest that although B10 and C11 are members of the same gene family and share nucleotide and amino acid sequence homologies, they appear to be regulated differently. They are targets for distinct as well as common factors which participate in the formation of complexes that are responsible for their CTL-specific and sequential expression.

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ABBREVIATIONS

ADCC:	antibody-dependent cellular cytotoxicity
AGP7:	azurophil granule protein 7
AP1:	activator protein-1, member of the jun-related protein family that binds to the sequence TGATCATCA in eukaryotes and is activated by phorbol esters
AP2:	activator protein-2 which mediates transcriptional activation in response to two signal transduction pathways: those involving protein kinase C and A.
APC:	antigen presenting cell
APRT:	adenine phosphoribosyltransferase
ATP:	adenosine triphosphate
bp:	base-pairs
$C\alpha$ or $C\beta$:	constant gene segments of immunoglobulin or T cell receptor alpha or beta chain genes
C3:	complement component C3
C9:	late complement component C9
C3b:	digestion product of complement protease C3
C3R:	complement C3 receptor
cAMP:	cyclic adenosine monophosphate
CAP37:	cationic antimicrobial protein of 37 kilodaltons
cat:	chloramphenicol acetyltransferase
CCAAT box:	eukaryotic gene promoter element with the consensus sequence GGt/cCAATG located ~70 bp upstream from the transcription start (+1)
CCPs:	cytotoxic cell proteases
CD2, 3, 4, 8, 16, 28, 56:	cluster of differentiation (T cell surface markers)
cDNA:	complementary DNA; DNA which has been copied from messenger RNA using the enzyme reverse transcriptase
C/EBP:	CCAAT/enhancer binding protein

CHO:	Chinese hamster ovary cells
CLE1,2:	conserved lymphokine element 1,2
conA:	concanavalin A; a plant lectin which is capable of stimulating T cells to proliferate and differentiate
cRABP:	cellular retinoic acid binding protein
CRE:	cAMP responsive element
CREB:	cAMP responsive element binding protein
CTF:	CCAAT-binding transcription factor
CTLs:	cytotoxic T lymphocytes
CTMC:	connective tissue mast cell
D:	diversity gene segments of immunoglobulin heavy chain or T cell receptor beta chain locus
DAG:	diacylglycerol
DCR:	dominant control region
DEAE:	diethylaminoethyl
DFP:	diisopropylfluorophosphate
DHFR:	dihydrofolate reductase
DMSO:	dimethyl sulfoxide
DNA:	deoxyribonucleic acid
DNAse1:	deoxyribonuclease 1
EDTA:	ethylenediamine tetraacetic acid
EGTA:	ethyleneglycol-bis-(beta-aminoethylether)n,n,n',n'-tetraacetic acid
ER:	endoplasmic reticulum
Ets-1:	eukaryotic nuclear protein with amino acid homology to the product encoded by the avian E26 leukemia *v-ets* oncogene
Fc fragment:	parts of immunoglobulin which have sites for activation of complement and for the binding of immunoglobulin to macrophages and polymorphonuclear granulocytes
G0, G1:	phases of the cell cycle; G0: cell division phase, G1: period between cell division and the start of DNA synthesis

Gal4/gal80:	transcriptional activator (gal4) and repressor (gal80) of genes required for galactose catabolism in the yeast
GC box:	A GGGCGG repeating element found in the promoter of many genes
G-CSF:	granulocyte colony stimulating factor
GM-CSF:	granulocyte-macrophage colony stimulating factor
GR:	glucocorticoid receptor
GRE:	glucocorticoid response element
GRP:	glucose-regulated proteins
HF:	Hanukah factor; alternative protein name for granzyme A
HIV-1:	human immunodeficiency virus type 1
HMG:	high-mobility-group
HSF:	heat shock factor
HSP70:	heat shock protein of 70 kilodaltons
HSV tk:	herpes virus thymidine kinase
ICAM-1:	intercellular adhesion molecule-1
IDDM:	insulin-dependent diabetes mellitus
Ig, IgG:	immunoglobulin, 5 classes IgG, A, M, D, E
IgH:	immunoglobulin heavy chain
IκB:	inhibitor associated with NFκB transcription factor
IL1, 2, 6:	interleukins 1, 2 and 6
IL2R:	interleukin 2 receptor
IP3:	inositoltriphosphate
kb:	kilobases
kbp:	kilobase pairs
kDa:	kilodaltons
LAK:	lymphokine-activated killer cells
lck:	lymphoid cell kinase

LCMV:	lymphocytic choriomeningitis virus
LFA-1, 3:	lymphocyte function-associated protein 1 or 3
LGL:	large granular lymphocytes
LT:	lymphotoxin
LTR:	long terminal repeat; a segment of DNA which is repeated at each end of the proviral form of a retrovirus
MCS:	multiple cloning site
mdr-1:	multidrug resistance-1
MHC:	major histocompatibility complex
MMCP-1, 2, 4:	mucosal mast cell protease 1, 2, or 4
MMTV:	mouse mammary tumor virus
MoMSV:	mouse Moloney sarcoma virus
mRNA:	messenger RNA
MT-1:	metallothionein-1
MTOC:	microtubule-organizing center
NFAT-1:	nuclear factor of activated T cells-1
NFIL2A:	nuclear factor IL2 A
NFκB:	nuclear factor kappa B
NK:	natural killer cells
NKCF:	natural killer cytotoxic factor
NOD:	nonobese diabetic mice
Oct-1, 2:	octamer binding protein-1, or 2
PBS:	phosphate buffered saline pH 7.2
PCRP:	phosphorylcholine, calcium-dependent receptor molecule for the lymphocyte perforin
pCTL:	precursor cytotoxic T lymphocyte
PEG:	polyethylene glycol
PI:	phosphatidylinositol

PMA:	phorbol-12-myristate-13-acetate; a phorbol diester compound capable of acting as a tumor promoting agent
PMSF:	phenylmethylsulfonylfluoride
poly(A):	polyadenylation signal or tail
RHFM:	tissue culture medium containing RPMI 1640, hepes buffer, fetal calf serum and β-mercaptoethanol
RMCPI, II:	rat mast cell protease I, or II
RSV:	Rous sarcoma virus; DNA tumor virus
SAR:	scaffold attachment regions
snRNAs:	small nuclear RNAs
SP1:	transcriptional factor that binds to the GC boxes of the SV40 early promoter
SV2, 40:	Simian sarcoma virus 2, and 40; DNA tumor virus
TAT:	tyrosine aminotransferase
TATA box:	eukaryotic gene promoter element with the consensus sequence TATAAA located 20 to 30 bp upstream from the transcription start site (+1)
TCR:	T cell antigen-specific receptor
TCR/CD3:	T cell receptor associated with the CD3 complex
TFIIA to F:	RNA polymerase II TATA box-binding transcription factors A to F
TGFβ or α:	transforming growth factor beta or alpha
TNF:	tumor necrosis factor
TNFα:	tumor necrosis factor alpha or TNF
TNFβ:	tumor necrosis factor beta or lymphotoxin
TPA:	12-O-tetradecanoyl phorbol-13-acetate, more commonly referred to as PMA
TRE:	TPA responsive element

CHAPTER I

INTRODUCTION

The present thesis centers on the mechanism of action of cytotoxic T lymphocytes (CTLs). Specifically, the work described focuses on the regulation of expression of two members of the family of CTL-specific proteases that are secreted during an encounter with a cellular invader. The goals of the introductory chapter are three fold: 1) to familiarize the reader with the various cellular components of the immune system, 2) to present a critical review of the more recent models of cell-mediated cytolysis and 3) to discuss current concepts in eukaryotic gene regulation and how such regulatory mechanisms impact on T cell development and activation.

A. AN OVERVIEW OF THE IMMUNE SYSTEM

1. *An intricate network of interactions*

Lymphocytes are active participants in the process of immunosurveillance. They protect an individual throughout its life from a myriad of foreign substances such as viruses, bacteria, fungi, parasites and even the body's own cells which have acquired foreign characteristics due to infection or malignant transformation. They do so by accurately and specifically recognizing non-self molecules, also termed antigens (structures of foreign origin capable of inducing an immune reaction), and destroying them. These specialized cells achieve their full protective potential through collaboration with less specialized cells in the body through direct cell-cell contact or indirectly via secreted factors. An elaborate communication system is in place between the various components of the immune system so the right decision is made upon encounter with an antigen to obtain full protection and allow the species to survive and to be preserved. Unlike the primitive immune system of lower animals, the vertebrate immune system exhibits memory; thus the system offers a long term protection by remembering the antigen encountered and responding faster and more efficiently upon reexposure. The past decade has provided exciting information on this complex set of interactions and the roles and mechanisms of action of some of the key players.

2. *Participants in an immune response*

The mammalian immune system can be separated into two branches, the antibody-mediated or humoral immunity and cell-mediated immunity (Figure I.1). B lymphocytes (producers of antibodies) mediate humoral immunity and they interact with antigens via specific immunoglobulin (Ig) receptors (Blackwell and Alt, 1988). T lymphocytes are responsible for cellular immunity and bind to antigens on cell surfaces via specific T cell receptors (TCR) (Davis, 1988). Antigens recognized by T cells are processed so that the determinant contacted by the T cell antigen receptor is only a small fragment of the original antigen presented in association with major histocompatibility complex (MHC) molecules (discussed in detail in section A.3).

Both the B and T cell antigen receptors have distinct structures (Figure I.2). The Ig receptors (and antibody molecules) are comprised of two identical light (L) and two identical heavy (H) polypeptide chains joined by disulphide bonds. There are two types of light chains, kappa (κ) and lambda (λ). At the amino termini of H and L chains are regions of highly variable amino acid sequence (V regions) that are paired with each other to form the site which binds antigen. The constant region of the H chains, from which the Fc fragment is derived, triggers effector functions such as complement fixation. It also represents the feature that distinguishes each of the five classes of immunoglobulins (IgG, IgA, IgM, IgD and IgE) which are comprised of the heavy chains γ, α, μ, δ and ε, respectively. The Ig receptors are usually referred to as B cell antigen receptors i.e. receptor proteins with antibody activity. Antibodies, on the other hand, correspond to the secreted immunoglobulins that combine with antigens in solution. The TCRs are

FIGURE I.1 Origin of the cellular components of the immune system. All cellular participants are derived from pluripotent stem cells that originate from the bone marrow. These stem cells give rise to two lineage-specific progenitor cells which in turn generate myeloid and lymphoid precursors which, under the influence of protein signals, develop step by step into a mature cell type. Polymorphonuclear granulocytes and monocytes originate from the myeloid progenitor cells while B, T and NK cells derive from the lymphoid stem cells. Differentiation of B cells takes place in the bone marrow and that of T cells into T killer, T helper and T suppressor subsets in the thymus. B cells are the producers of antibodies and as such are involved in humoral immunity. T killer lymphocytes destroy cellular invaders and are referred to as the effectors of the immune system. T helper and T suppressor cells control or regulate immune responses and are seen as the regulators. The three T cell subsets comprise cellular immunity.

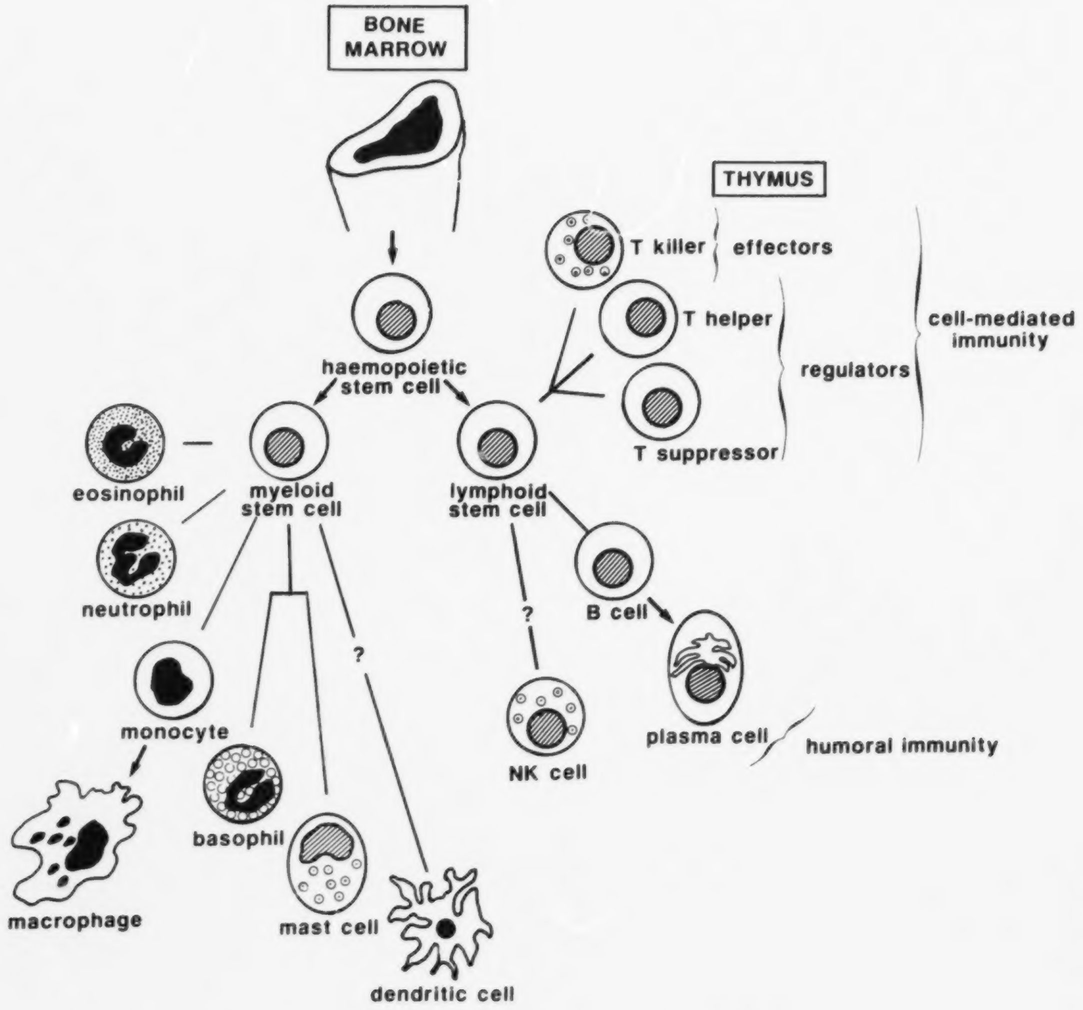
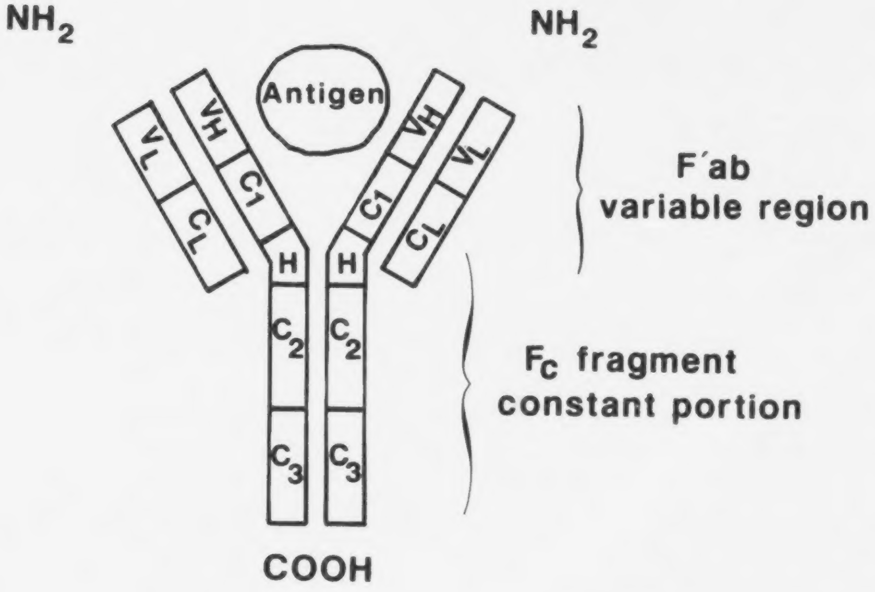
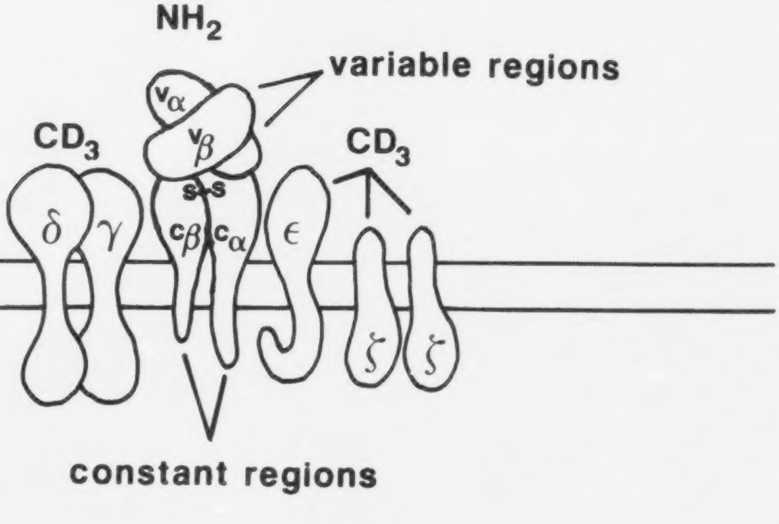

BONE MARROW
THYMUS
haemopoietic stem cell
myeloid stem cell
lymphoid stem cell
T killer
effectors
T helper
T suppressor
regulators
cell-mediated immunity
eosinophil
neutrophil
monocyte
macrophage
basophil
mast cell
?
dendritic cell
?
NK cell
B cell
plasma cell
humoral immunity

FIGURE I.2 The basic structures of a typical antibody (immunoglobulin G) and T cell receptor molecules. A. The amino terminal end of IgG is characterized by sequence variability (V) in both the heavy (H) and light (L) chains which are referred to as the V_H and V_L regions respectively. The rest of the molecule has a relatively constant (C) structure. The constant portion of the light chain is termed the C_L region. The constant domain of the heavy chain is further divided into three structurally discrete regions: C1, C2 and C3. These globular regions, which are stabilized by intrachain disulphide bonds, are referred to as "domains". The sites at which the antibody binds antigen are located in the variable domains. The hinge region designated H allows changes in the distance between the two antigen binding sites and permits them to operate independently. B. The antigen-specific T cell receptor most commonly found (in 95% of T cells) is a disulphide-linked heterodimeric glycoprotein comprised of an α and a β chain. Antigens bind to the variable domains of α and β polypeptides at the amino terminus of the molecule represented by Vα and Vβ. In the membrane of a T cell, the T cell receptor is closely associated with the CD3 δ, γ, ε and ζ molecules. On some T cells, the T cell receptor γ and δ chains replace those of α and β.

A.


NH2
NH2
Antigen
VL
VH
C1
CL
H
C2
C3
VH
VL
C1
CL
H
C2
C3
F´ab
variable region
Fc fragment
constant portion
COOH

B.


NH2
variable regions
vα
vβ
CD3
CD3
s-s
δ
γ
cβ
cα
ϵ
ζ
ζ
constant regions

heterodimers comprised of α and β or γ and δ glycoprotein chains linked via disulphide bonds and closely associated with other molecules (known collectively as CD3). As in the Ig polypeptides, the amino termini of the TCR chains are polymorphic and responsible for binding antigens.

Both the Ig receptors and TCRs are made up of polypeptides that are encoded by a large genetic locus which features variable (V), diversity (D), joining (J) and constant (C) gene segments that are rearranged during lymphocyte differentiation. The incredible diversity of Ig receptors and TCRs arises from the random utilisation of a large number of germline V, D, and J gene segments, from the junctional variation (insertion and/or deletion of nucleotides) generated between VD, DJ or VJ gene segments during the joining process and from the random association of α and β or γ and δ chains to give the final products. B and T lymphocytes which represent the major participants in an immune response are described here along with other important cellular components.

2.1 B cells and their origin

B cells or antibody-forming cells derive from immature precursors in the bone marrow through a process of antigen-independent maturation. It is during their differentiation that lymphocytes become committed to bind antigen through rearrangement and expression of antigen-specific immunoglobulin receptor genes (Warner et al., 1974, Tonegawa et al., 1983). Following these critical events, mature B cells migrate to various lymphoid organs where they are found in clusters called lymphoid follicles. These mature B cells differentiate to antibody-secreting cells called plasma cells upon interaction with cognate antigen via their Ig receptor and influenced by the presence of factors produced by macrophages or T cells. Plasma cells secrete antibodies with the same specificity for antigen as that found in the receptor of the B cell from which it derived. Each B cell reacts with a unique antigenic determinant, i.e. all the receptors on a B cell are monospecific. The secreted antibodies enter the blood and other fluids of the body to defend against soluble factors produced by bacterial or viral infections.

2.2 T cell subsets and their origin

T cells arise from pluripotent stem cells located in the bone marrow (Ford et al., 1966). These T cell precursors then migrate to the thymus where they mature and differentiate into functional T cells (T helper, T killer and T suppressor; see below) (Scollay et al., 1984). It is during this maturational step that rearrangement and expression of genes encoding the antigen receptor takes place. The majority of the mature T cell population is comprised of cells expressing the α/β heterodimeric receptors and 1-5% expressing the γ/δ TCR genes (Pardoll et al., 1987, Strominger, 1989). Following rearrangement of the antigen receptor genes, T cells are subject to selective mechanisms that lead to the survival of cells able to respond to foreign antigen and the death or inactivation of cells recognizing self-antigen (Scollay et al., 1980, Davis, 1990).

After these critical events, the T lymphocytes leave the thymus and migrate to the secondary lymphoid organs where they remain quiescent until they come into contact with their cognate antigen. Upon interaction with antigen, they are driven through the final steps of maturation and acquire immunologic functions.

2.2.1 Helper T cells

When the body is under attack helper T cells sound the alarm. As their name implies, they regulate or help other cells of the immune system: B cells to secrete antibody and cytotoxic T cells to become functional. Stimulated helper T cells release glycoproteins known as lymphokines that have profound effects on other cells, including the lymphocytes themselves. Some factors result in the recruitment of other cellular components to the site of attack and in their activation, regulation and deactivation (reviewed in Mosmann and Coffman, 1989). Helper T cells usually express the CD4 glycoprotein on their cell surface.

This molecule interacts with class II MHC glycoproteins found on the surface of a limited number of specialized cells termed antigen-presenting cells (B cells, macrophages). Helper T cells are able to recognize antigens only in association with class II MHC molecules and thus are said to be class II MHC-restricted.

2.2.2 Cytotoxic T cells

These cells represent the most specific weapon available to the immune system for the eradication of virus-infected and tumor cells. In addition, they are directly involved in the rejection of grafts during the course of unsuccessful transplantations (reviewed in Nabholz and MacDonald, 1983 and Colvin, 1990). CTLs are generated within 4 to 9 days after viral infection. By 14 days there is a decrease in their activity, but they then maintain a state of immunological memory and readily become reactivated in response to reinfection by the same virus. CTLs play a dual role: 1) they bind to abnormal or virus-infected cells, destroy them and stop the production and spread of new viral particles 2) following their interaction with the abnormal or virus-infected cells, they secrete interferon-γ which stimulates the production of activated macrophages. Interferon-γ also induces a state of antiviral activity in the cells of the host (Pasternack, 1988, Colvin, 1990). The majority of killer T cells are $CD8^+$ and thus recognize antigens in association with class I MHC molecules that are found on most cells in the body. Their activation and mechanism of action are discussed in section B.

2.2.3 Suppressor T cells

These cells function to limit and terminate the responses of other lymphocytes. They are particularly important in tuning down the immune response when the battle is over (Ferguson et al., 1988). They are stimulated concurrently with the appearance of helper T cells and the development of the response to antigen. Suppressor T cells with specificity for T cells, B cells, and even antigen-presenting cells have been reported (Horvat et al., 1989).

Although several putative suppressor lymphokines have been identified and partially characterized (Ferguson et al., 1988), the mechanism(s) of action of the suppressor T cells remain(s) to be determined. Clearly these cells could potentially play a dominant role in regulation of immune response.

2.3 Macrophages

Macrophages or mononuclear phagocytes are derived from myeloid precursors found in the bone marrow (Figure I.1). These precursors, the promonocytes, enter the blood and become monocytes which circulate for a day or two before migrating randomly to different tissues where they mature to macrophages (Abramson et al., 1977). These highly phagocytic cells are widely distributed throughout the body in blood, bone marrow, liver, lymphoid tissue, connective tissue and nervous tissue. They form an important part of the defense mechanism by removing microorganisms through phagocytosis (the process by which particulate materials are bound and engulfed) and pinocytosis (a process whereby soluble molecules are taken up) (reviewed in Unanue and Allen, 1987). Macrophages swallow a wide variety of antigens via specific cell surface receptors Fc and C3 which recognize materials coated with antibodies and complement, respectively. The Fc receptors bind specifically to the heavy chain of IgG (Unkeless et al., 1981). The C3 receptor recognizes C3b protein from the complement (Rabellino et al., 1978). Macrophages also contain numerous lysosomes and endocytic vesicles. The lysosomes contain a large variety of lytic enzymes with specificity for proteins, polysaccharides, lipids and nucleic acids which make the process of intracellular digestion extremely efficient. The action of the activated macrophage is non-specific; that is to say, the macrophage will inactivate bacteria or virus regardless of the organism that induced the infection.

Besides the functions of pinocytosis, phagocytosis and intracellular digestion, the phagocytes are active secretory cells that synthesize and secrete a large number of materials (Unanue, 1976, Unanue and Allen, 1987). Among these are enzymes capable of affecting extracellular proteins (plasminogen activators, collagenase, elastase, lysosomal proteases), products involved in defense processes (lysosome, complement proteins, interferon-α) and factors capable of modulating the function of surrounding cells (interleukin-1 (IL1), IL6 and tumor necrosis factor (TNF)), or growth factors stimulating the proliferation of specific types of cells (granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF) and transforming growth factor β (TGFβ)). The range of secretory products of phagocytes attests to their obvious importance in defense mechanisms. They provide to both B and T cells the extracellular signals required for their functional activation.

2.4 Polymorphonuclear granulocytes

Neutrophils, eosinophils, basophils and mast cells derive from myeloid precursors found in the bone marrow (Figure I.1). They all participate in the inflammatory response mediated by antibodies and complement (Gleich and Adolphson, 1986, Weiss, 1989, Gordon et al., 1990). None of these cells show specificity for antigens but are still crucial for the eradication of microorganisms. The predominant role of polymorphs is phagocytosis. They have receptors for Fc fragments of IgG or IgE and for C3b, which enable them to engulf opsonized (antibody-coated or complement-coated) organisms. They also contain primary, azurophilic granules rich in myeloperoxidase. This enzyme, together with hydrogen peroxide generated during the respiratory burst that takes place after phagocytosis, are involved in oxidative mechanisms responsible for bacterial killing (Bentwood and Henson, 1980).

Besides their involvement in phagocytosis, the granulocytes also play more specialized roles. They are frequent cellular components in allergic reactions and are implicated in defense against parasites (Gleich and Adolphson, 1986, Gordon et al., 1990). The interaction of parasite or allergen (such as pollen) with effector cells (eosinophils, basophils or mast cells) via the Fc receptors induces their degranulation which results in the release of some very basic proteins (in the case of eosinophils) that are lethal for the parasites and in the release of heparin, serine proteases and mediators of anaphylaxis (in the case of basophils and mast cells) which cause symptoms of allergy but also play a role in immunity against the microorganisms. This type of reaction is the only way that these cells can use their "granule armament" against large targets which cannot be phagocytosed. Eosinophils also release histaminase and aryl sulphatase which inactivate the mast cell products histamine and slow reactive substances of anaphylaxis, respectively. They in a sense regulate the inflammatory response by limiting the granulocyte migration into the site of invasion.

2.5 Natural killer cells

Like CTLs, natural killer (NK) cells have the ability to destroy tumor cells *in vitro* and *in vivo* (Trinchieri, 1989, Lotzová, 1990). Studies in a number of different laboratories suggest that IL2-*in vitro* stimulated NK cells are responsible for the lymphokine-activated killer (LAK) phenomenon observed in tumor-bearing hosts in which solid tumors are prevented from metastasizing (Rosenberg, 1988, Lotzová and Herberman, 1987). NK cells can be easily identified morphologically as large granular lymphocytes (LGL) with $CD3^-$, $CD16^+$, $CD56^+$ cell surface phenotype. This characteristic combination of surface differentiation antigens serve to define them as a discrete and homogeneous leukocyte subset distinct from both B and T cells and myelomonocytic cells. NK cells are quite broadly distributed in different tissue compartments, which include peripheral blood, spleen, liver, lungs, peritoneal cavity and intestine-associated tissues. NK cells unlike CTLs, are not MHC-restricted and therefore are capable of lysing a variety of targets. They possess receptors for the Fc fragment of immunoglobulin and can thus recognize and kill cells coated with antibodies. This antibody-dependent cell cytotoxicity (ADCC) is also a property of macrophages and granulocytes in addition to NK cells.

NK cells can also bind and kill some tumor or virus-infected cells in the absence of antibody through a process involving degranulation (Carpén and Saksela, 1988, Atkinson et al., 1990). The molecular basis of the binding and recognition by NK as well as their mechanism of killing will be discussed in detail in section B. As potent mediators of both spontaneous and antibody-dependent cytotoxicity and as key players in the elimination of malignant-tumor cells, NK cells appear to complement the antigen-specific CTLs.

3. *T cell activation*

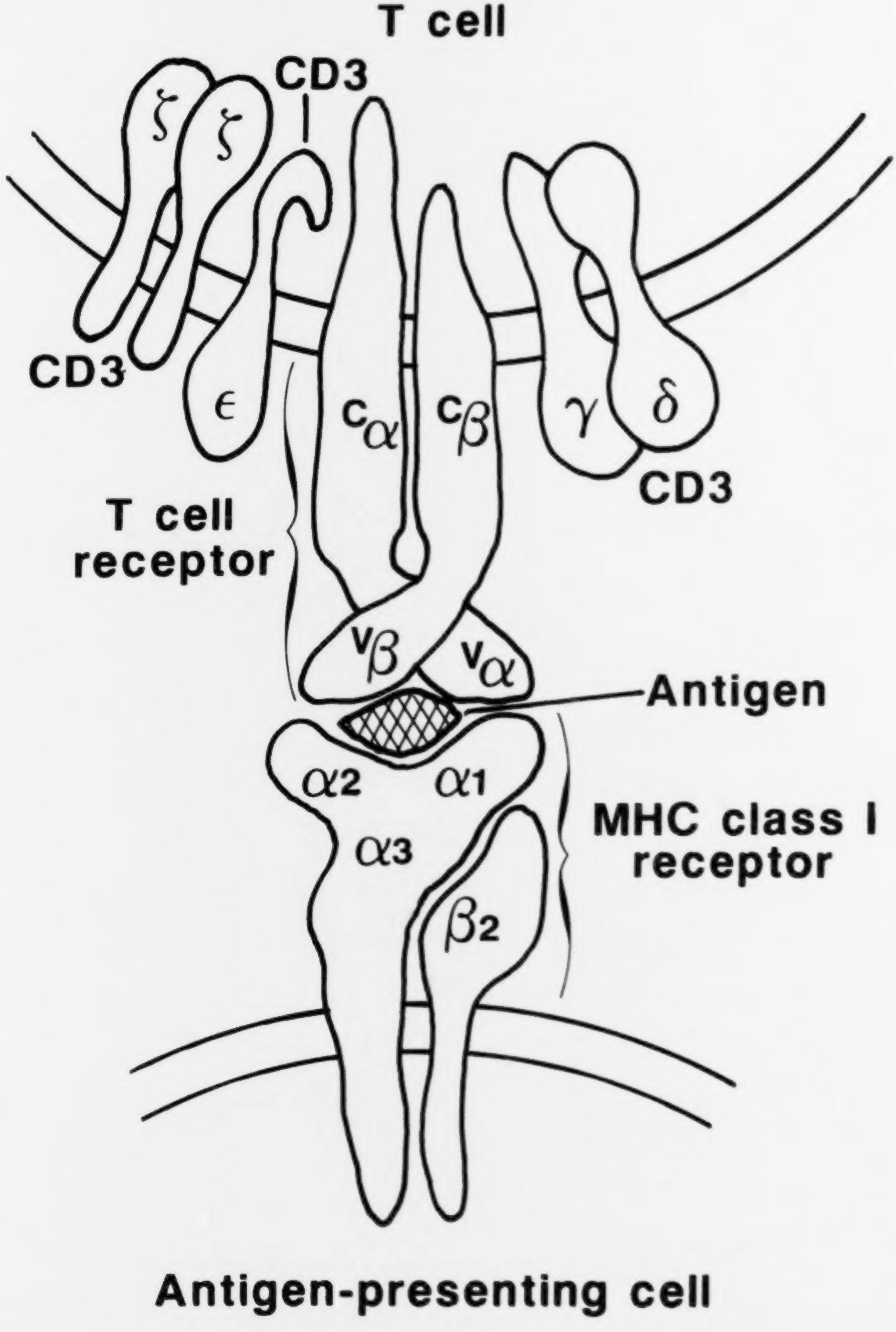
The activation of T cells from their quiescent or resting state can be divided into two steps: 1) antigen recognition and 2) cell proliferation and establishment of effector function. These last two steps are intimately linked and are rarely presented as two separate stages.

3.1 Antigen recognition

The antigen-specific T cell receptor (TCR) and the major histocompatibility complex (MHC) glycoproteins are the key elements of specificity in the T cell response to foreign antigens (Townsend and Bodmer, 1989, Kumar et al., 1989). TCRs recognize short, linear peptide determinants of 10-20 amino acids, the generation of which usually requires unfolding and proteolytic fragmentation (processing) of the antigenic protein. In addition, this antigenic peptide must be bound by an MHC glycoprotein and it is this complex of MHC molecule plus peptide that forms the structure recognized by the TCR. MHC glycoproteins are thus peptide-binding proteins, and can be considered as antigen-presenting molecules (Bjorkman and Parham, 1990).

The structure of class I MHC molecules consist of one glycosylated polypeptide chain (45 kDa) noncovalently associated with a nonglycosylated peptide termed β2-microglobulin (12 kDa) (Figure I.3). The MHC-encoded chain has three globular domains (termed α1, α2 and α3). The α3 domain is conserved and is closely associated with the non-MHC-encoded peptide β2-microglobulin. X-ray crystallographic studies have

FIGURE I.3 A diagrammatic representation of the trimolecular complex formed upon antigen recognition by T killer lymphocytes. T killer cells recognize antigens only in association with MHC class I molecules. The polymorphic domains of the T cell receptor (Vα and Vβ) contact antigens bound within the groove made from the α1 and α2 variable domains of MHC class I. Cα and Cβ represent constant regions of the T cell receptor. α1, α2, α3 and β2 show the three α chain regions and β2-microglobulin of the class I MHC complex. The CD3 γ, δ, ε and ζ glycoproteins that are closely associated with the T cell receptor are also shown.


T cell
CD3
ζ
ζ
CD3
CD3
ϵ
cα
cβ
γ
δ
CD3
T cell receptor
vβ
vα
Antigen
α2
α1
α3
MHC class I receptor
β2
Antigen-presenting cell

demonstrated that α1 and α2 domains constitute a "platform" that consists of an eight-stranded β-pleated sheet topped by two long α-helices that lie diagonally across the sheet. Several lines of evidence suggest that the binding site for antigenic peptides is a deep groove located between the two long α-helices (Bjorkman and Parham, 1990). Because of the depth of the binding site, one side of a peptide bound to MHC would always be easily accessible to a TCR (Davis, 1990, Bjorkman and Parham, 1990). Thus, antigen-recognition by a TCR implies making direct contact with side chain atoms from the MHC molecule as well as the peptide. The detail of the recognition process are still unclear however modelling experiments have suggested that antigen recognition by TCR is similar to that described for antibodies. Basically, the TCR variable domains fold into tertiary structures similar to antibody V regions which form a groove where the antigen can bind (Figure I.3). The limited diversity in specific regions on TCR Vα and Vβ contacts the side chains of the MHC α-helices, leaving the centrally located and very diverse regions of Vα and Vβ to interact with the peptide. Like the antigen-antibody complex, a better understanding of the recognition process will come after the analysis of crystal structures of a specific TCR alone or as a complex with its peptide-MHC ligand.

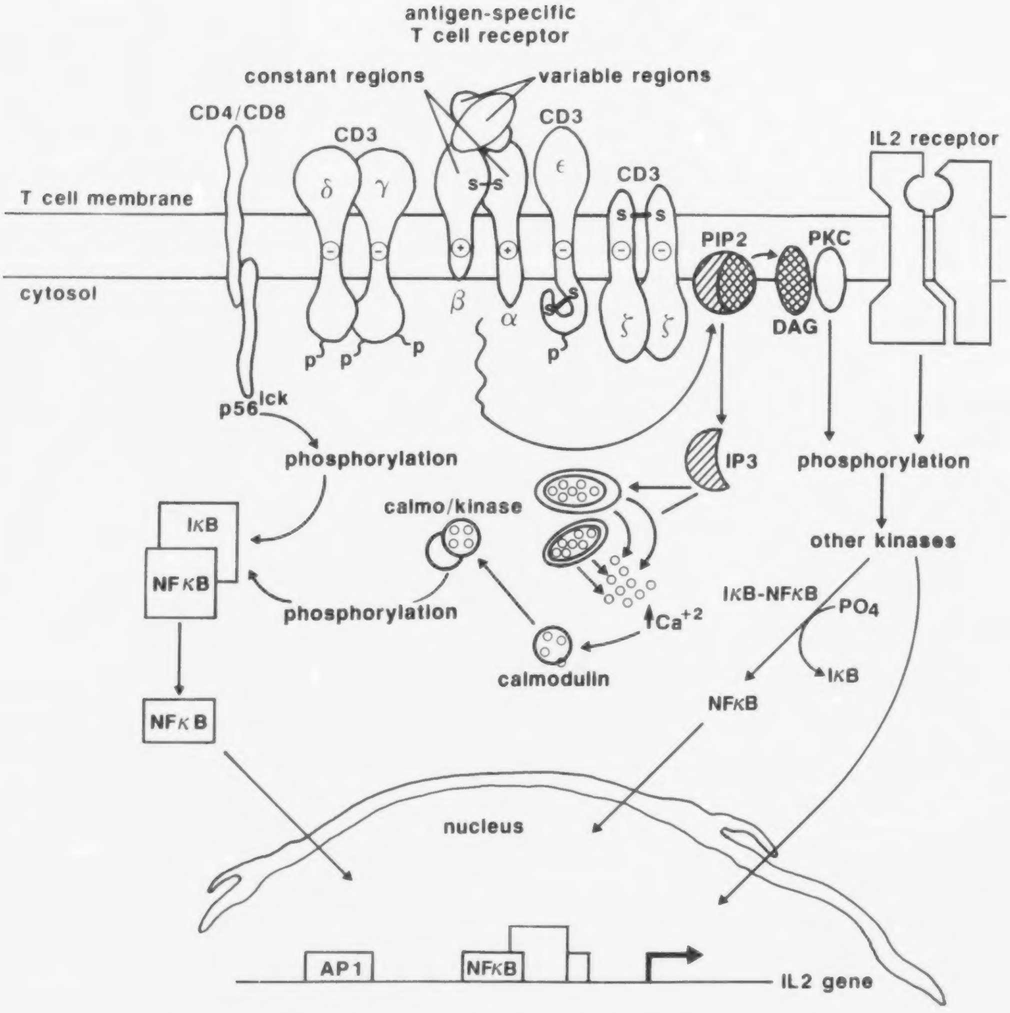
Recent data have provided convincing evidence that CD4 and CD8 are MHC receptors with CD4 specifically able to bind class II molecules (Doyle and Strominger, 1987) and CD8 directed to class I MHC molecules (Normant et al., 1988). How this is accomplished is not certain however it is clear that the presence of CD4 or CD8 allows the recognition process to proceed more efficiently by increasing the avidity with which a T cell binds to its antigen-bearing cell (Gabert et al., 1987, Sleckman et al., 1987). CD4 and CD8 may also promote the interaction of the TCR with its appropriate antigen. Other accessory molecules such as CD2 have been identified through the use of monoclonal antibodies which participate in increasing intercellular adhesion (Bierer et al., 1989).

3.2 Cell activation

For both helper T cells and killer T cells, the α/β heterodimeric receptor is assembled and expressed together with four distinct CD3 molecules, namely γ, δ, ε and ζ (Davis, 1990). Interaction between the TCR/CD3 complex and antigen associated with an MHC product on the antigen-presenting cell initiates transmembrane signalling by activating phospholipase C which hydrolyzes the membrane component phosphatidyl 4,5-bisphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG) (Figure I.4). IP3 causes the release of calcium ions from intracellular stores, resulting in a transient rise in cytosolic calcium (Ca^{2+}). The ions bind to calmodulin, and the calcium-calmodulin complex activates protein kinases which phosphorylate cytosolic proteins (such as IκB, inhibitor of the transcription factor NFκB). This phosphorylation step liberates NFκB from the complex and allows it to reach the nucleus where it participates in the activation of genes such as IL2. The Ca^{2+} released from intracellular compartments and the increase in DAG also prompt the protein kinase C to associate with the membrane. This activation brings about the phosphorylation and activation of a different set of proteins which could include other kinases. These, in turn, could activate still other enzymes to modify the function of many different target proteins such as IκB releasing NFκB and permitting its translocation in the nucleus. Another possibility exists where kinases themselves enter the nucleus and act on transcription factors or other nuclear proteins. The tyrosine kinase intrinsic to the IL2 receptor can also participate in the phosphorylation events and activate factors implicated in the transcriptional regulation of specific genes. The phosphorylated substrates thus participate in transduction of the activation signal into the nucleus.

Evidence that the four distinct CD3 molecules are involved in signal transduction came from experiments showing that antibody directed at CD3 components can stimulate T cells in the same way as antigen (Davis, 1990, Terhorst, 1988). Furthermore, it was shown that during the activation of T cells, all of the CD3 polypeptides are phosphorylated at

FIGURE I.4 A model of signal transduction pathways during antigen-mediated T cell activation. Interaction between the TCR/CD3 complex on T cells and antigen associated with an MHC product on the antigen-presenting cell initiates transmembrane signalling by activating phospholipase C which hydrolyzes the membrane component phosphatidyl 4,5-bisphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 induces the release of calcium ions from intracellular compartments, resulting in an increase in calcium (Ca^{2+}). The ions bind to calmodulin, and the calcium-calmodulin complex activates protein kinases which phosphorylate cytosolic proteins (such as IκB, inhibitor of NFκB transcription factor). This phosphorylation step liberates NFκB from the complex and allows it to reach the nucleus where it participates in the activation of the IL2 gene expression. The presence of an increased amount of Ca^{2+} and DAG stimulates the association of the enzyme pkC (protein kinase C) with the membrane. The activated pkC then phosphorylates different sets of proteins which may include other kinases. These, in turn, may activate enzymes which modify the function of other target proteins such as IκB. Another possibility exists where kinases themselves enter the nucleus and act on transcription factors or other nuclear proteins. The T cell-specific receptors CD4 and CD8 also transduce signals through their distinct kinase ($p56^{lck}$) and can promote phosphorylation of other proteins.


antigen-specific
T cell receptor
constant regions
variable regions
CD4/CD8
CD3
CD3
CD3
IL2 receptor
T cell membrane
cytosol
δ
γ
ε
s-s
s-s
s-s
β
α
ζ
ζ
p
p
p
p
PIP2
PKC
DAG
p56lck
phosphorylation
IP3
phosphorylation
IκB
NFκB
calmo/kinase
other kinases
phosphorylation
IκB-NFκB
PO4
Ca+2
IκB
calmodulin
NFκB
NFκB
nucleus
AP1
NFκB
IL2 gene

tyrosine or serine residues (Samelson et al., 1986, Cantrell et al., 1985). Very recently, two additional polypeptides have been found that coprecipitate with CD3 molecules thus bringing the number of polypeptides comprising the TCR/CD3 complex to nine (Fraser et al., 1989).

In addition to their clear ability to agglutinate T cells with MHC^+ specific targets, CD4 or CD8 has also been implicated in signalling effects (Figure I.4). Recently a 56 kDa tyrosine kinase (lymphoid cell kinase [lck]) was found associated with each molecule (Veillette et al., 1988, Rudd et al., 1988). More recently, Abraham and Veillette (1991) demonstrated that $p56^{lck}$ positively regulates T cell functions by transducing CD4/CD8 triggered tyrosine phosphorylation signals during antigen stimulation. Their results were confirmed by Glaichenhaus et al. (1991). Whether these signals are qualitatively distinct from those initiated by the TCR and/or amplify signals initiated by the TCR using similar pathways of activation is unknown.

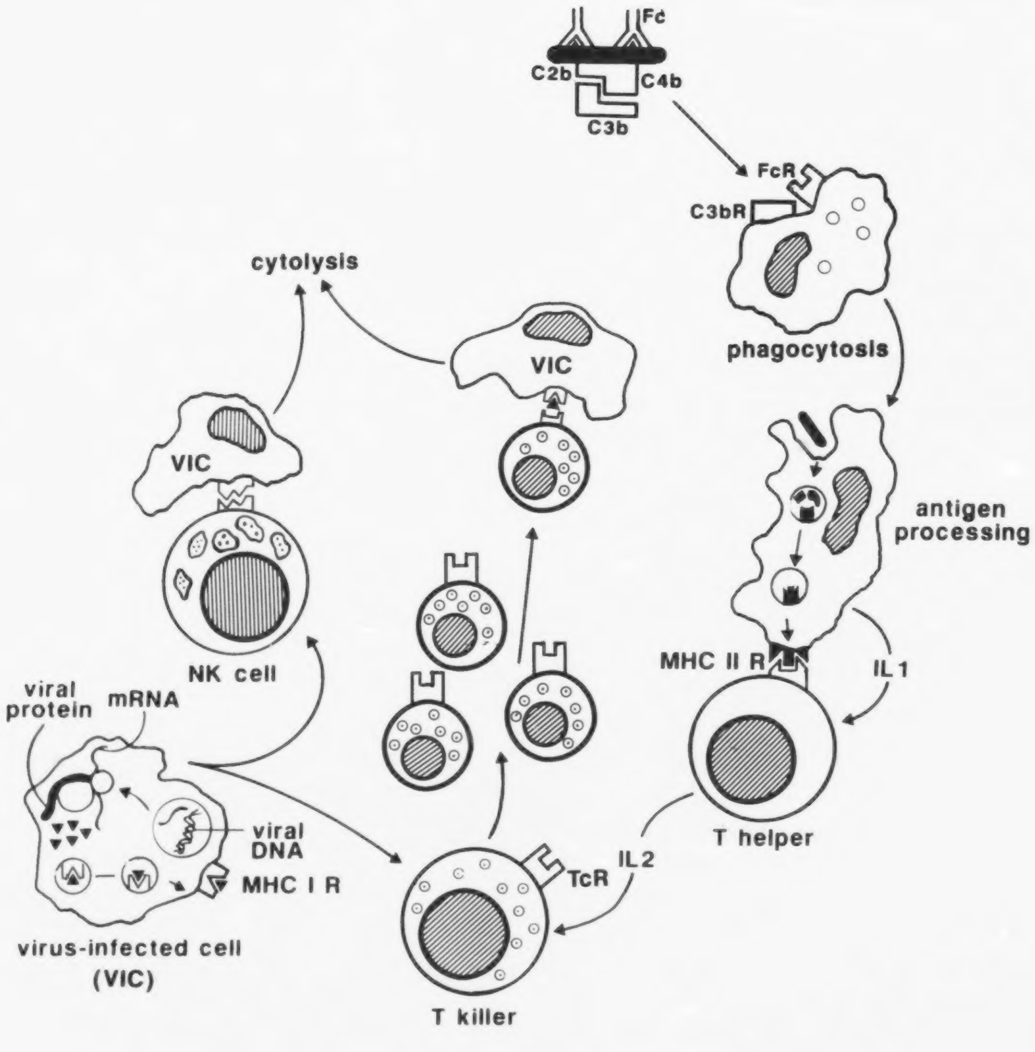
The series of biochemical reactions that are triggered by the transduced signals and take place in the nucleus serve to move both precursors of T helper and T killer cells from G0 into the G1 stage of the cell cycle (Crabtree, 1989, Isakov et al., 1987). During this activation phase, new mRNA and proteins are synthesized in each T cell subset. For stimulated T helper cells, genes that encode IL2 and the IL2 receptor α chain are activated. The newly synthesized subunit of the IL2 receptor associates with the subunit already on the cell surface. Together, they have a high affinity for IL2, while the constitutive chain alone comprises the low affinity receptor. The subsequent interaction of IL2 with its high affinity receptor promotes transit through the remainder of the cell cycle resulting in clonal expansion of the cells originally stimulated by antigen. Binding of IL2 to its receptor results in enhanced secretion of lymphokines, increased expression of membrane receptors for other growth factors (transferrin receptor, insulin receptor) and the expression of induced histocompatibility molecules.

For the antigen-stimulated precursor CTL (pCTL), the burst in transcription results in synthesis of new proteins that include one subunit of the IL2 receptor, the transferrin receptor, c-myc and MHC class I molecules. Up-regulation of the transferrin receptor is necessary since transferrin is a universal requirement for long-term growth of all mammalian cell types (Cotner et al., 1983). The protooncogene c-myc is a nuclear DNA binding protein which seems to contribute to entry of the pCTL into S phase during activation. The association of the newly synthesized IL2 receptor α chain with the constitutive IL2 receptor β subunit gives rise to a high affinity receptor on the pCTL which bind the lymphokine produced by the neighboring T helper cells. This interaction induces progression to the second stage of cell activation in which DNA synthesis takes place and the cells proliferate into a clonal population of cytotoxic cells expressing the same specific T cell antigen receptor. These cells are now competent to bind and lyse target cells.

The intracellular signalling events induced by IL2 were recently examined (Saltzman et al., 1990). It was found that the binding of IL2 to its respective high affinity receptor does not stimulate calcium mobilization or increase production of phosphatidylinositol (PI) metabolites. Thus, the pathways by which lymphokine signals are relayed to T lymphocytes do not appear to involve the classical second messengers diacylglycerol (DAG) and inositoltriphosphates (IP3). Very recently, Eardley and Koshland (1991) observed that IL2 bound to its receptor initiates the rapid hydrolysis of an inositol-containing glycolipid to yield a myristylated diacylglycerol in the membrane and an inositol phosphate-glycan into the cytoplasm via the activation of a specific phospholipase C. It was suggested that the tyrosine kinase associated with the IL2 receptor could be involved in activation of the phospholipase C (see Figure I.4). Presumably, some of the events induced by this activation would include phosphorylation reactions which have a direct impact on gene activation.

In summary, T cell activation can be viewed as follows (Figure I.5): macrophages present antigens to helper T cells and provide a source of IL1 which augments the production of IL2 and induces expression of the IL2 receptor on antigen-activated helper T

FIGURE I.5 Network of interactions between the cellular components of the immune system. Foreign antigen (bacteria) coated with antibodies (IgG) activate the complement system by binding C1. One component of the complement cascade C3b binds to the C3b receptor on the surface of macrophages and induces phagocytosis of the microbes. The macrophages (antigen-presenting cells) break down the foreign proteins and display the pieces together with MHC class II surface proteins. At the same time, macrophages secrete interleukin-1 (IL1) growth factor. T helper cells recognize the displayed antigen/MHC II complex and are activated to produce IL2, important for T killer lymphocytes. Concurrently, abnormal proteins made within a virus-infected or malignant cell are degraded in the cell's cytoplasm. Only then do the fragments move into a vesicle where they join class I MHC molecules and are carried to the cell surface for display to T killers. Following interaction between TCR/CD3 of the T killer and MHC I/antigen receptor on the invader cell in the presence of IL2 from neighboring T helper cells, T killer lymphocytes are primed to lyse target cells. NK cells interact with their targets via unknown receptors. Following this association, target cells are programmed for destruction.


Fc
C2b
C4b
C3b
FcR
C3bR
cytolysis
phagocytosis
VIC
VIC
antigen processing
NK cell
MHC II R
IL1
viral protein
mRNA
viral DNA
MHC I R
TcR
IL2
T helper
virus-infected cell (VIC)
T killer

cells. The secretion of IL2 by helper T cells is accompanied by the production of a variety of lymphocyte differentiation and growth factors. These in turn activate macrophages and increase their phagocytic activity and activate CTLs and induce their proliferation into cytotoxic clones. T cell activation proceeds following a dual signalling event: helper T cells require both IL1 from macrophages and antigen presented by MHC class II, killer T cells need both IL2 from helper T cells and antigen presented by MHC class I and macrophages require both antigen and lymphokines produced by both helper T and killer T cells.

B. MECHANISMS OF KILLING

As mentioned previously, CTLs and NK cells are particularly prominent in killing tumor and virus-infected cells and are also largely responsible for graft rejection. For over a decade, considerable effort has been expended trying to understand the mechanism(s) by which these cells destroy their targets. Some of the major discoveries that have contributed to our understanding of cell-mediated cytolysis are reviewed in this section.

Several strategies have been used to investigate the mechanisms of cytotoxicity. One of these was to fractionate components of the cell and test their cytotoxic activity *in vitro* (Henkart, 1985, Young, 1989). Another approach was to isolate specific transcripts that are expressed only in CTLs, as these should include genes that encode key compounds of the cytolytic machinery. This was achieved by making cDNA libraries from CTL and other lymphocyte subsets and using differential and subtractive hybridization to identify those messages expressed only in CTL (Bleackley et al., 1988, Gershenfeld and Weissman, 1986, Lobe et al., 1986a,b, Brunet et al., 1986, Kwon et al., 1987, Jenne et al., 1988). Both methods have provided interesting information that has been formulated into models.

1. *Common features of cell-mediated cytolysis models*

Although the models presented may differ in their actual mode of action, they share common features. They all divide the process of cytolysis into four distinct stages: 1) recognition of and binding to the target cell, 2) delivery of the lethal hit, 3) detachment of the effector cell from the target, and 4) death of the target cell. The various models differ in step two: one model suggests that the lytic molecules are located within intracellular granules of the effector cells whereas the others indicate that target cell destruction is mediated by molecules not localised in granules. It is probable that multiple mechanisms of cytolysis exist perhaps even within the same cell. Although all the current models are described, the emphasis of this section is on the granule-mediated killing model and the granule-localized components. The serine proteases analysed in this thesis are believed to be active participants in this cytolysis model.

1.1 Recognition and binding to the target cell

In all the models proposed there is a requirement for close contact between the lymphocyte and the target cell, that takes place within a few minutes (Hiserodt et al., 1982). The initial contact is made nonspecifically through two CTL-cell adhesion molecules found on the surface of lymphocytes, LFA-1 (lymphocyte function-associated protein 1) and CD2 (cluster of differentiation 2) that interact with the two target cell surface proteins called ICAM-1 (intercellular adhesion molecule 1) and LFA-3 respectively (Springer et al., 1987). This magnesium-dependent binding process is strengthened by specific interactions between the TCR/CD3 complex on the CTL and a combination of its cognate antigen and MHC class I molecules on the target cell. The avidity of the cell-cell contacts is enhanced further by the binding of CD8 to determinants of MHC class I molecule on the target cell (Terhorst, 1988, Bierer et al., 1989). The precise role of these nonspecific adhesion molecules in killing by CTLs is unclear. However, their importance was underscored by transfection experiments showing that cells bearing antigen recognized by CTLs were not lysed unless nonspecific conjugate formation occurred (Spits et al., 1986). Recent studies suggest that these accessory molecules may also play a role in the transduction of activation signals from the TCR/CD3 complex through the cytoplasm into the nucleus where genes that encode the molecules responsible for the effector function of CTLs are induced (Bierer et al., 1989).

1.2 Reorganization of the internal organelles of the effector cell

Binding of the target cell leads to a dramatic reorganization of the CTL which can be seen as the polarization of the microtubule-organizing center (MTOC) and the Golgi apparatus toward the target cell, followed by movement of granules toward the area of contact (Kupfer et al., 1985, Kupfer and Dennert, 1984). Immunofluorescent staining reveals that in the killer cell both actin and tubulin but not myosin are polarized toward the target (Geiger et al., 1982). A role for cytoplasmic granules and their reorientation during cell killing has also been substantiated by direct visualization of cell conjugates using high-resolution Nomarski optics cinematography. Using this technique, Yannelli et al. (1986) were able to monitor continuously the interaction between a CTL and its target cell. The nucleus of the killer cell was seen to be displaced away from the target cell soon after target binding, and granules migrated toward the site of cell-cell contact.

1.3 Detachment of the effector cell from the target

After delivery of the lethal hit, the killer cell may dissociate from the target without interrupting the lytic process. The effector may then initiate a new lytic cycle with another target cell.

1.4 Target cell lysis

Several dramatic changes are observed in the target cell during the lytic phase. A massive calcium influx and changes in the overall shape of the target cell are followed by expansion of the ER and chromatin condensation (Duvall and Wyllie, 1986). These morphologic changes are accompanied by DNA fragmentation, and ultimately, because of cytoplasmic membrane damage, the release of macromolecules into the extracellular environment. The DNA is cleaved into discretely sized fragments that each differ by approximately 200 base pairs, apparently because of the activation of an endogenous nuclease (Russell and Dobos, 1980, Duke et al., 1983). The nature of the signal(s) triggered by lymphocytes that would initiate this program of cell death remain(s) to be determined. The dual disintegration of DNA and plasma membrane is clearly different from the events observed in complement-mediated lysis, during which DNA fragmentation is not observed (Russell et al., 1980).

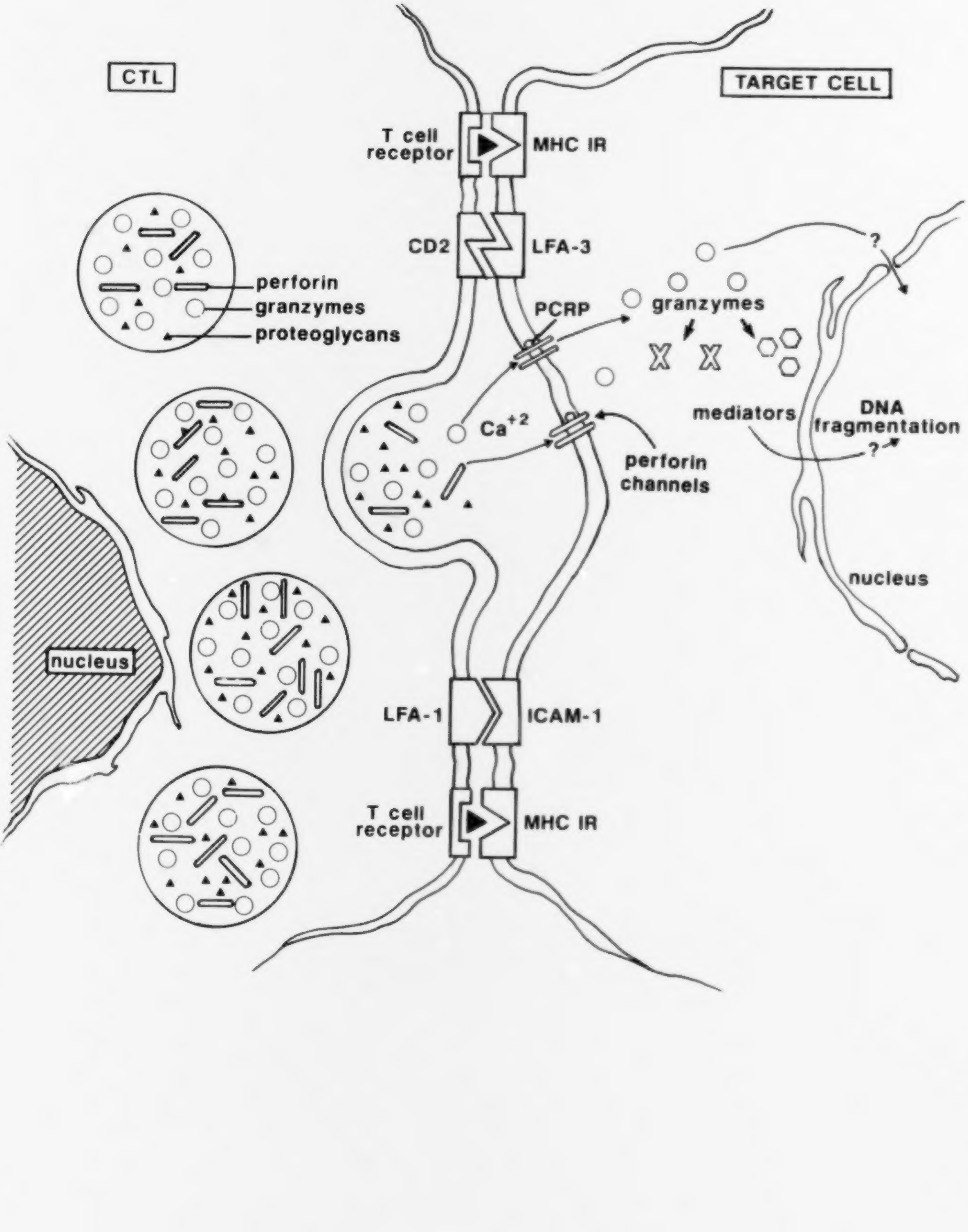
2. *Delivery of the lethal hit*

2.1 Granule-exocytosis model

In the most popular model to date, degranulation accompanies delivery of the lethal hit. Following recognition and binding to a target cell, the effector cell degranulates, releasing lytic proteins such as perforin and serine proteases into the immediate vicinity of the target membrane (Figure I.6). In the presence of calcium, perforin polymerizes and forms polyperforin channels through which lytic proteins can gain access to the interior of the target cell and cause cell damage (reviewed in Joag et al., 1989, Young, 1989, Krähenbühl and Tschopp, 1990). The characterization of perforin, serine proteases and other candidates for lytic proteins implicated in this model are described later in this section.

Early studies carried out by several groups showed that calcium is necessary for delivery of the lethal hit during a stage in which the target cell is programmed to undergo cell death. Others demonstrated that the engagement of the TCR by antigen binding is reportedly followed by rapid increases in cytosolic calcium levels (Gray et al., 1987). Moreover, stimulation of CTLs with a combination of calcium ionophores and phorbol esters is known to trigger the CTL to kill. More recent reports indicated that the perforin pathway is absolutely calcium-dependent (Tschopp and Nabholz, 1990). Agents that reduce calcium

FIGURE I.6 Granule exocytosis or pore formation model of lymphocyte-mediated killing. The cytotoxic T lymphocyte (CTL) binds to its target (virus-infected or transformed cell) via nonspecific interactions mediated by CD2/LFA-1 on the CTL membrane and LFA-3/ICAM-1 on the target surface. These cell-cell contacts are further strengthened by highly specific binding of the TCR with MHC class I antigen complex on the target cell (TC). The triggering of the TCR/CD3 complex and CD8 receptor causes an increase in Ca^{2+} ions which promotes degranulation following reorientation of the Golgi apparatus towards the point of contact with the TC. The granule lytic components are released in the interspace between the CTL and TC. Perforin polymerizes in the presence of Ca^{2+} and associates with the phosphorylcholine in the target membrane (PCRP) to form pores. These allow other lytic proteins such as CCPs (granzymes) to penetrate the target and reach the nucleus to activate an endonuclease which fragments the TC DNA. CCPs may also activate mediators in the TC cytoplasm which in turn would induce the TC endonuclease and promote cell death.


CTL
TARGET CELL
T cell receptor
MHC IR
CD2
LFA-3
perforin
granzymes
proteoglycans
PCRP
granzymes
?
Ca+2
mediators
DNA fragmentation
?
perforin channels
nucleus
nucleus
LFA-1
ICAM-1
T cell receptor
MHC IR

levels (EGTA, EDTA) were shown to inhibit killing of target cells (Hiserodt et al., 1982, Henney, 1973, Joag et al., 1989). These studies have collectively implied that both signalling and the effector mechanisms of CTLs are strictly calcium-dependent. Other drugs that interfere with degranulation or exocytosis also inhibit killer cell activity. They include colchicine, a disrupter of microtubule organization; chloroquine, a weak base that localizes selectively in lysosomes and endosomes, increasing their pH; and monensin, which interrupts membrane traffic through the Golgi apparatus.

2.2 Alternative mechanisms of target cell lysis

Recent observations in several laboratories have established that some CTL cell lines can kill target cells in the absence of calcium and perforin or serine proteases (Trenn et al., 1987, Ostergaard et al., 1987, Ostergaard and Clark, 1989). These observations challenged the concept that degranulation was a prerequisite for killing. Tirosh and Berke (1985) had shown in an earlier study that some target cells require calcium for lysis, whereas others do not. A recent report indicated that, depending whether primary or cultured CTL populations were used as effectors, different calcium requirements were seen (Howell and Martz, 1988). These findings were provocative in suggesting that different mechanisms of cytolysis may be involved in the lysis of target cells by CTLs and that the pathway of lysis employed depends on both the effector CTL and the target populations used. It is now well accepted that the various postulated killing mechanisms are not mutually exclusive and that they may operate singly or in concert, depending on the conditions used to elicit cytolytic cells (Ostergaard and Clark, 1989, Young, 1989, Tschopp and Nabholz, 1990).

2.2.1 The internal target disintegration mediated by elevated cytosolic calcium levels

Berke and Rosen (1988) suggested that CTL populations that have been primed *in vivo* do not use the perforin pathway to kill. Rather, CTLs induce within minutes of lymphocyte-target cell interaction, an increase in cytosolic calcium from internal or external stores which ultimately leads to cytolysis (Tirosh and Berke, 1985). Their model implicates the increase in calcium levels as the first event in target cell death. There is evidence to suggest that massive and persistent increases in cytosolic Ca^{2+} may contribute to DNA fragmentation by triggering calcium-dependent topoisomerases and nucleases, which induce uncoiling and fragmentation of the DNA, respectively (Berke, 1989). In addition, it was suggested that target cell protease activity, induced or enhanced by Ca^{2+}, could damage cytoskeletal elements, resulting in bleb formation and finally zeiosis (cell boiling). Energy production was also suppressed by Ca^{2+}-induced damage to mitochondria, thus affecting the function of ATP-fueled ion pumps.

Basically, the cytolysis model that they proposed include four steps: 1) early depolarization of the target cell membrane stimulated by the CTL, 2) cytosolic mobilization of Ca^{2+} in the target cell, either by influx through voltage-dependent channels or by internal release, 3) exhaustive activation of ATP hydrolysis mainly by the Ca^{2+}-regulated actomyosin system, 4) colloid-osmotic swelling and cell lysis as a result of ATP depletion impairing osmotic regulation by active ion pumping.

Although this model does not involve degranulation and release of perforin or serine proteases, it is not in disagreement with the granule-exocytosis model since perforin insertion into the target cell membrane was shown to induce a rapid increase in cytosolic Ca^{2+} as one of the steps leading to lysis (Nabholz and Tschopp, 1989).

2.2.2 CTL-induced target cell death through secretion of high concentrations of extracellular ATP

Extracellular ATP profoundly effects cellular functions as well as cell membrane properties and intracellular biochemical reactions (Gordon et al., 1986). Filippini et al. (1990a,b) suggested the possibility that extracellular ATP acting in concert with the cell-surface ATP-receptors and/or ectoprotein kinases leads to the CTL-induced target cell death.

They demonstrated that stimulation of the CTL T-cell antigen receptor results in the extracellular Ca^{2+}-independent accumulation of ATP in the interspace between the antigen-bearing target cell and the CTL which ultimately leads to target cell death. The same authors identified ecto-ATPase receptors on CTLs which are believed to protect CTLs from lytic effects of the high local concentrations of ATP. Further work is required to verify the validity of this proposed model.

2.2.3 Target cell lysis induced by soluble mediators

This model is based on a family of cytolytic proteins with functional and immunological relationships to tumor necrosis factor (TNF,TNFα or cachectin) (Beutler and Cerami, 1989). In addition to TNF itself, the family includes lymphotoxin (LT or TNFβ) (Young, 1989), leukalexin (Liu et al., 1987) and natural killer cytotoxic factor (NKCF) (Wright and Bonavida, 1982, 1987). These molecules are found abundantly in the cytosol of CTLs and NK cells and are believed to be delivered to the surface of the target cell where they associate with specific receptors (Young and Liu, 1988b). Following this interaction, the target cell is programmed to undergo cell death.

To date, the role of these cytokines in lymphocyte-mediated killing is unclear. They induce lysis in hours rather than minutes as seen with cytolysis mediated by cytotoxic lymphocytes (Henney, 1975, Henkart, 1985, Young, 1989, Wright and Bonavida, 1987). In addition, if a soluble mediator was being secreted into the milieu, then lysis should eventually be independent of cell-cell collisions, which, as numerous studies have shown, it is not. These observations argue against a role for these proteins in cell-mediated killing.

3. *Resistance of killer lymphocytes to their own cytolytic machinery*

How CTLs kill target cells but are themselves spared from being lysed during the cytolytic event has remained largely an unresolved matter (Young and Liu, 1988a, Müller-Eberhard, 1988). Several laboratories have shown that cytolytic lymphocytes are equipped with the means to protect themselves from killing by other cytolytic lymphocytes, by extracted granules and by purified perforin (Kranz and Eisen, 1987, Blakely et al., 1987). In the case of granule-mediated killing, experiments attempting to clarify this issue showed that 1) cytolytic cell lines do not protect themselves from lysis by preventing the killer cell from releasing lytic granule components (Kranz and Eisen, 1987, Verret et al., 1987, Nagler-Anderson et al., 1988) 2) the protective phenomenon does not involve a rapid removal of lesions or the activation of ion pumps and other energy-dependent processes that restore electrochemical gradients. Therefore the protective phenomenon appears to be extremely specific. More recently, Jiang et al. (1990) demonstrated the presence of a protective protein in the membranes of CTLs which interferes with perforin binding to the CTL surface.

However, other groups have indicated that under certain circumstances, CTLs can be killed by other CTLs (Walden and Eisen, 1990, Pemberton et al., 1990, Joag et al., 1989). Further investigations are required to resolve this controversial issue.

4. *Molecules associated with granules*

The direct involvement of granules in lymphocyte-mediated killing was demonstrated primarily by studies which showed that granules isolated from CTLs and NK cell clones were cytolytic (Henkart et al., 1984). Many studies have been devoted, in the past several years, to the biochemical characterization of CTL-derived granules, and as a result, several components have been purified and identified.

4.1 Perforin

The first direct structural evidence for pore formation on target cell membranes was only reported in 1980 (Dourmashkin et al., Henkart et al.). They showed, by electron microscopy, that ring-like lesions were acquired on antibody-covered erythrocyte ghosts after attack by lymphocytes. These studies revealed tubular structures with an internal diameter of 15 nm. Podack and Dennert (1983) later confirmed and significantly extended these findings to cloned CTLs and NK cells. Both cytotoxic cell types are capable of assembling two types of tubular lesions, one with an internal diameter of 16 nm, named polyperforin 1 and a second small lesion with an internal diameter of 5 nm, polyperforin 2. The monomer was named perforin for its ability to perforate membranes (Podack, 1985).

Perforin (also called pore-forming protein (Liu et al., 1986) or cytolysin (Henkart, 1985) or C9-related protein (Zalman et al., 1986)) has now been isolated from mouse, rat, human CTL, NK or LGL granules and has a monomer molecular mass of 65-75 kDa (Tschopp and Nabholz, 1990). Both CTL and NK cells appear to contain the same perforin species based on their identified molecular mass and functional and structural properties. Perforin lyses targets nonspecifically but only in the presence of calcium. Calcium induces the polymerization of the perforin monomer into supramolecular structures that resist dissociation by detergents, disulfide-bond reducing reagents and boiling. Complete circular polymerization is not obligatory for functional channel formation.

Perforin was originally believed to induce cytolysis by forming channels in the target cell membrane resulting in colloid-osmotic lysis (Henkart and Henkart, 1982, Berke, 1985). However, it is becoming increasingly clear that perforin alone cannot account for all of the observed effects of target cell death (see below) (Russell, 1983, Hayes et al., 1989, Zychlinsky et al., 1991). In this regard, one interesting aspect of the pore formation model is that these transmembrane channels might allow other granule components to gain access to the interior of the target cell where they could exert their lytic potential. Candidates for such lytic molecules include a family of granule-associated serine proteases referred to as cytotoxic cell proteases (CCPs) or granzymes.

4.2 Cytotoxic cell proteases or granule-localized proteases (granzymes)

Considerable evidence for the involvement of cellular proteases in cell-mediated cytotoxicity has accumulated during the past several years. Early studies determined that various serine protease inhibitors such as diisopropylfluorophosphate (DFP), phenylmethylsulfonylfluoride (PMSF) and macromolecular protease inhibitors (such as α1 antitrypsin) were capable of blocking CTL- and NK-mediated killing (Matter, 1975, Hudig et al., 1984, Ferluga et al., 1982, Hudig et al., 1981). In addition, it was shown that CTL lines contain much higher levels of serine protease activity (based on a trypsin-sensitive substrate) than noncytolytic cells (Pasternack and Eisen, 1985). Recently, a number of CTL-specific cDNAs were cloned and shown to encode serine proteases (Lobe et al., 1986a,b, Gershenfeld and Weissman, 1986, Brunet et al, 1986, Bleackley et al., 1988, Jenne et al., 1988). Most importantly, transcription activation of these genes correlates with the development of cytolytic activity (Bleackley et al., 1988) and the proteases themselves are in granules (Redmond et al., 1987).

Following these studies, several serine proteases were isolated from the granules of CTLs and NK cells and shown to be released during cell killing. To date, six homologous serine proteases, termed CCPs or granzymes have been found in the cytoplasmic granules of murine CTLs (see Table I.1) (Mason and Tschopp, 1987, Prendergast et al., 1991, Bleackley et al., 1988, Jenne et al., 1989, Haddad et al., 1991). An additional granule protease Hanukah factor (HF) (granzyme A) has also been characterized but it is not recognized as a member of the CCP family because it has a different structure, gene organization and chromosomal location from the others. These proteolytic enzymes comprise approximately 85% of the proteins present in the granules of mouse CTL lines (10% for perforin). In human CTL or lymphokine-activated killer cells, however, there is so far only evidence for the presence of four proteases, three of which have been characterized at the genetic level. These are the human homologues to murine Hanukah factor (HuHF) and CCP1 (HuCCP1) and another related serine protease HuCCPX. The relationship of the fourth human CCP is still unclear (Prendergast et al., 1991, Meier et al., 1990, Fruth et al., 1987, Schmid and Weissman, 1987, Hameed et al., 1988, Gershenfeld et al., 1988).

Clone (DNA sequence) Designation	Protein Name	Alternative Protein Name
C11	CCP1	granzyme B
B10	CCP2	granzyme C
D12	CCP3	granzyme E
c134	CCP4	granzyme F
11-15	CCP5	granzyme D
13.4	CCP6	granzyme G
AR10	HF	granzyme A

TABLE I.1 A summary of the CTL-specific protease genes and the proteins they encode. The cytotoxic cell protease (CCP) designation is that of Lobe et al. (1986), Bleackley et al. (1988), granzymes have been characterized by Masson and Tschopp (1987), Jenne et al. (1988 and 1989), Haddad et al. (1991). Hanukah factor (granzyme A) was described by Gershenfeld and Weissman (1986).

CCPs are very similar to each other in primary structure (at least 55% identity) and contain the His, Asp, Ser triad known to form the catalytic center of serine proteases (Bleackley et al., 1988, Prendergast et al., 1991, Jenne et al., 1989, Haddad et al., 1991). Also conserved is the hallmark Asp-Ser-Gly-Gly motif that includes the active site serine. The mature proteases all begin with the common amino-terminal sequence Ile-Ile-Gly-Gly following a pre-propeptide. All CCPs contain the acidic dipeptide Gly-Glu or Glu-Glu (propeptide) following the signal peptide (hydrophobic prepeptide) which directs secretion or intracellular localization (ER). After signal peptide removal, the acidic amino-terminal dipeptide of CCPs is cleaved during assembly of the granules to yield the mature protease.

The CCP family members all exist as monomers that are comprised of many basic residues such as arginine, histidine and lysine. Furthermore, all CCPs lack a particular disulfide bridge normally present in chymotrypsin or trypsin (two of the classical proteases characterized). This bridge is responsible for the stability of the substrate binding site.

Again HF is an exception because in addition to having four disulfide bridges, it occurs as a disulfide-linked homodimer. The enzymatic properties of some of the CCPs have also been determined. CCP1 preferentially hydrolyses synthetic substrates after Met, Ser and Asp (Odake et al., 1991). CCP5, on the other hand, has a weak trypsin-like activity and cleaves after Lys or Arg residues (Jenne and Tschopp, 1988). The specificity of the remaining CCPs (2, 3, 4, 7) is still unclear as no proteolytic activity was measured when they were tested on a variety of synthetic substrates (Tschopp and Nabholz, 1990). These observations imply that CCPs may bind to and cleave highly specific substrates in a very restricted fashion. Masson et al. (1986) examined the proteolytic properties of the granule protease HF and showed that it has a trypsin-like activity as indicated by the aspartic acid residue in the binding pocket and by its substrate specificity for Arg-X bonds. It cleaves among other proteins, fibrin, casein and extracellular matrix proteins.

The function of the proteases remains speculative at the present time (Kramer and Simon, 1987, Tschopp and Nabholz, 1987). Unlike perforin, purified CCPs alone have no cytolytic activity suggesting that these enzymes play an intermediate processing role that may be required to render perforin and other lytic mediators active (Young, 1989). CCPs might cleave proteoglycans bound to perforin (see below) and thereby activate this pore-forming protein. Indeed, all CCPs share a high degree of homology with cathepsin G which has been shown to degrade proteoglycans (Jenne et al., 1989, Hanson et al., 1990). It has also been suggested that CCPs may be important in facilitating the detachment of CTLs from the target cell thereby allowing the CTLs to recycle i.e., bind to and lyse additional targets (Tschopp and Nabholz, 1987). Purified HF can stimulate growth of B cells and thymic lymphoma cells (Simon et al., 1987). In addition, it can degrade endothelial-derived extracellular matrix proteins and thus may help the migration of activated lymphocytes into tissues (Simon et al., 1987).

Targets of NK and CTL action undergo an early fragmentation of chromosomal DNA, presumably via activation of a target cell endonucleolytic system. As mentioned previously, CCPs could use perforin channels to reach the interior of the target and initiate damage by inducing an endonuclease enzyme for example (Munger et al., 1988). Supporting this view, Hayes et al. (1989) reported recently that granule-associated protease activity co-purifies with "nuclear DNA-releasing" activity, and that a combination of granule-associated perforin and serine proteases could induce both target cell lysis and significant DNA release. Controversial reports have been published however, which demonstrate that cytolytic granules isolated from CTL clones do not induce DNA degradation (Gromkowski et al., 1988, Duke et al., 1988). Therefore, it is still unclear whether serine proteases play an active role in this type of cell death. Further work is necessary to elucidate their biological role.

Serine proteases have also been detected in the cytoplasmic granules of other specialized cells of the immune system. Normal human monocytes and neutrophils contain cathepsin G and elastase which upon release, allow the rapid degradation of extracellular substrates (Kargi et al., 1989, Weiss, 1989). This in turn promotes transvascular migration of cells, tissue remodelling during morphogenesis, wound healing, inflammatory reactions and a number of pathologic conditions including tumor invasion. In addition, at least four other granule proteases have been identified in neutrophils (Wilde et al., 1990, Kao et al., 1988, Pereira et al., 1990). The 37 kDa cationic antimicrobial protein CAP37, proteinase 3 and azurocidin share sequence similarity with elastase. Azurophil granule protein 7 (AGP7), on the other hand, is 70% identical to CCP1 (within the first 20 amino acids from the amino-terminal end).

Mast cells which have been implicated in the expression of a wide variety of biological responses also contain large amounts of serine proteases within the secretory granules (Benfey et al., 1987, Serafin et al., 1990). In both the mouse and rat, the connective tissue mast cell (CTMC) serine protease is designated RMCPI (also named chymase) whereas the mucosal mast cell enzyme is termed RMCPII. Both enzymes resemble pancreatic chymotrypsin in that they are serine endopeptidases that cleave to the carboxy terminal side of hydrophobic amino acids with a neutral to basic pH optimum. RMCPI and RMCPII have substantial amino acid sequence homology with one another but are still distinct gene products. Recent studies have indicated that four additional distinct serine proteases are present in the secretory granules of the mouse CTMC (Serafin et al., 1991). Human mast cells have elaborated yet another kind of serine proteases termed tryptase (Vanderslice et al., 1990). Perhaps some clues regarding CCP function can be gleaned from studies on similar proteases in these nonlymphocytic cells.

4.3 Proteoglycans/ chondroitin sulfate A

Besides perforin and the family of proteases, the CTL and NK granules also contain chondroitin sulfate A proteoglycans. Schmidt et al. (1985) demonstrated that these negatively charged molecules are released from killer cells upon stimulation and prior to lysis of target cells. Although the function of the proteoglycans remains unclear, they have been attributed many roles. Serafin et al. (1986) presented evidence that they act as carrier and packaging molecules for proteolytic enzymes by interacting with the basic residues of the polypeptide chains. Indeed, CCPs and perforin have been shown to bind chondroitin sulfate glycoproteins. This observation further suggested that proteoglycans may have a protective role in preventing self-injury mediated by perforin and other lytic molecules by blocking their action in the granules in which they are stored (Tschopp and Masson, 1987). Another function which has been attributed to chondroitin sulfate A in CTLs is to increase the adhesion between the effector killer lymphocyte and its target cell upon its release from granules. This in turn would limit the space in which the lytic components can effect target cell damage.

5. *Gene organization of CCPs and perforin*

Full length genomic clones for most of the CCPs have been isolated very recently (Lobe et al., 1988, Prendergast et al., 1991, Jenne et al., 1989, Haddad et al., 1991). By comparison of the genomic and cDNA sequences, it was possible to determine the exon/intron organization of these genes. Each gene is encoded by five exons interrupted by four introns. The sizes of exons and introns for all members are comparable. Moreover, the positions of the introns are very well conserved in all CCP genes (Prendergast et al., 1991). Their genomic organization suggests that CCPs arose from a single primordial gene through gene duplication. Such an analysis also revealed that CCP2, 3, 4, 5 and 6 share the same 3'-untranslated sequences with CCP1 but have inherited different 5'-ends through the course of evolution. The homology at the nucleotide level is very high as summarized in Table I.2. The nucleotide identities are highest among CCPs 3, 4 and 6, ranging from 84% to 94%. The most closely related CCPs are CCP3 and CCP5, with 94% nucleotide identity. CCP1 and CCP2 genes which are the focus in the present thesis are 76% homologous at the nucleotide level and 55% identical at the amino acid level.

The CCP nucleotide sequences also share a high degree of homology with other members of the serine protease superfamily. The non-CTL proteases adipsin, cathepsin G, neutrophil elastase and rat mast cell protease II (RMCPII) contain sequences that are conserved in CTL-serine protease genes (Prendergast et al., 1991, Haddad et al., 1991, Vanderslice et al., 1990, Lobe et al., 1986a, Jenne et al., 1989). The serine protease domain of complement factor B, that of urokinase, the pancreatic proelastase II share overall 75% nucleotide identity with CCPs. For CCP1 and CCP2 genes, the greatest homology was found with the human cathepsin G gene with 65% and 62% nucleotide identity, respectively.

Five CCP genes have been assigned a chromosomal position. CCP1, 2, 3 and 4 genes have been localized to chromosome 14 in the mouse (Crosby et al., 1990, Klein et al., 1989, Brunet et al., 1986) and CCP1 and CCPX in human, to a region very close to the α T cell receptor locus (Meier et al., 1990). The HF gene maps to a totally different location (chromosome 5) which emphasizes the complex evolution of serine proteases (Gershenfeld et al., 1988).

	CCP1	CCP2	CCP3	CCP4	CCP5	CCP6	HF
CCP1		75	64	65	65	64	48
CCP2	66		69	70	70	70	48
CCP3	56	58		85	95	89	48
CCP4	57	59	76		84	86	47
CCP5	57	59	90	74		88	47
CCP6	58	61	82	79	78		46
HF	43	41	40	40	39	38	

TABLE I.2 Homologies between the known CTL-specific serine protease genes and proteins. The cDNA sequences corresponding to the coding portions of the genes were compared using the "Alignment" program of the Microgenie software package. Numbers above diagonal reflect the % nucleotide identities and numbers below diagonal represent % amino acid identities.

Recently, two groups have independently cloned mouse perforin cDNAs (Shinkai et al., 1988, Lowrey et al., 1989). A cDNA clone for human perforin and its genomic version have been isolated and sequenced (Lichtenheld et al., 1988, Lichtenheld and Podack, 1989). An interesting feature of human perforin is its organization in only three exons, one of which encodes almost the entire 5'-untranslated region. In addition, the distance between each exon is quite large (1663 bp for exon1 and exon2, 1182 bp for exon2 and exon3). The human perforin gene has been mapped to chromosome 17 (Shinkai et al., 1989).

6. *Transcriptional regulation of CCPs and perforin*

The interest in CTL-proteases grew much stronger when their distribution and kinetics of expression were examined. Transcription of CCP1, CCP2 and HF genes was detected specifically in stimulated cytotoxic T cells, NK cells, LAK cells and was not found in resting CTLs, resting or activated B cells, macrophages, brain, liver or fibroblasts (Lobe et al., 1986b, Bleackley et al., 1988, Velotti et al., 1989, Young et al., 1990, Gershenfeld and Weissman, 1986). γ/δ T cells which have been described in association with a number of immunologic disorders, also express CCP1 and HF (Koizumi et al., 1991). Moreover, during the development of a cytotoxic response *in vitro*, the steady-state level of mRNAs as well as the transcriptional rate of CCP1 and CCP2 were shown to parallel the cytolytic activity of the cells (Lobe et al., 1986b, Bleackley et al., 1988). These experiments also revealed that CCP1 and CCP2 were sequentially expressed and most importantly, that their level of mRNAs peaked 12 to 24 hours before the peak of cytotoxicity. This pattern of expression suggested that these mRNAs encoded proteins involved in cell-mediated killing. Very recently, the age-related decline in CTL activity has been correlated with an age-related decrease in serine protease mRNA level and activity (Bloom et al., 1990).

In every situation examined *in vitro*, transcription of the protease genes was always induced in response to stimulation of pCTL by antigen and lymphokines and correlated with cytolytic activity. Recently, Müeller et al. (1988a, 1988b, 1989) demonstrated the presence of serine protease transcripts in lymphocytes from cardiac allografts undergoing rejection, in a number of lymphocyte-associated dermatoses and in the brain or liver of lymphocytic choriomeningitis virus (LCMV)-infected mice. Finally, the gene for HF was shown to be expressed *in vivo* in islet-infiltrating cells during the development of autoimmune diabetes in NOD mice (nonobese diabetic mice which spontaneously develop a diabetic syndrome resembling human insulin-dependent diabetes mellitus (IDDM)) (Held et al., 1990). This set of results emphasizes the fact that expression of proteases occurs *in vivo* and is not an artefact of *in vitro* manipulation of cell lines.

The large body of biochemical information accumulated on perforin seem to indicate that this molecule plays a vital role in cytotoxicity and that pore formation is an integral part of lymphocyte-mediated reactions. Both murine and human forms of perforin are found predominantly in $CD8^+$ (CTL cells) and $CD16^+$ (Fc receptor marker-NK cells) phenotypes following *in vitro* stimulation with allogeneic cells (Young et al., 1990, Tschopp and Nabholz, 1990). It is absent from non-lymphocyte populations, from activated $CD4^+$ (helper T cells) and from resting $CD4^+$ and $CD8^+$ T cells. Human peripheral blood lymphocytes become potent killers and acquire perforin when stimulated with an anti-CD3 monoclonal antibody. Among *in vitro* cultured lymphocyte populations examined to date, there is a good correlation between cytolytic activity and the presence of perforin activity. Perforin has also been detected in CTLs raised *in vivo*. During an acute LCMV infection, massive numbers of CTLs with perforin have been detected in the brain or the liver of infected mice by *in situ* hybridization or staining with anti-perforin antibody (Müeller et al., 1989, Young et al., 1989). The same techniques were used very recently to show that perforin is expressed by lymphocytes found in the synovial joint fluid of patients with rheumatoid arthritis and in islets undergoing autoimmune destruction during IDDM (Young et al., 1989, Young et al., 1990).

In summary, despite rapid advances in the cellular and molecular characterization of CTLs and the elaboration of several interesting models, a clear picture of the mechanism(s) by which they effect target cell killing *in vivo* remains elusive.

The recent identification of the lytic effectors and the isolation of their genomic clones currently allows the study of the mechanism(s) which control their transcriptional activation. These analyses will provide clues on the nature of the intracellular signals which mediate activation of CTLs to the lytic state. Moreover, they will allow the delineation of the proteases and perforin essential regulatory genetic elements which could be used to design experiments to modulate the activity of these lytic molecules at will. Such studies will eventually help to define the role of these important players in cell-mediated lysis.

C. REGULATION OF GENE EXPRESSION

1. *General concepts of gene regulation in eukaryotes*

The processes of decoding genes and synthesizing appropriate amounts of gene products are complex. Numerous studies have shown that regulation can theoretically occur at any step in the biogenesis of mRNA: synthesis of the primary transcript (Struhl, 1989, Goodbourn, 1990, Maniatis et al., 1987), splicing (Smith et al., 1989, Maniatis, 1991), processing (addition of the 5' seven methyl G cap and 3' poly(A) residues) (Jackson and Standart, 1990, Proudfoot, 1991), transport from the nucleus to the cytoplasm (Wickens and Dahlberg, 1987, Zamore et al., 1990), and degradation of the mRNA, whether in the nucleus or cytoplasm (Wisdom and Lee, 1991, Shyu et al., 1991). Regulation can also take place at the translational (Craigen and Caskey, 1987, Riis et al., 1990) and/or post-translational level (Berk, 1989, Hershey, 1989). This section presents some of the general concepts of transcriptional regulation of gene expression in eukaryotes and purposely omits posttranscriptional and translational controls since these levels are beyond the scope of the work described herein.

Over the last ten years, the majority of research has concentrated upon the most common and immediate focal point of gene regulation, namely transcription. The conclusion of many studies indicated that for all genes examined, tissue-specific or stimulus-specific expression was conferred upon the gene by cis-acting sequence elements (DNA sequences linked to a gene). These genomic segments fall into three broad classes, namely promoters, enhancers and silencers and are composed of discrete DNA sequence motifs which serve as recognition blocks for sequence-specific DNA-binding factors. Positive and negative factors facilitate and prevent, respectively the assembly of transcription complexes which augment or restrict the recruitment of RNA polymerase II to the desired gene.

1.1 Promoters and initiation of transcription

Promoters are defined as the DNA sequences that allow RNA polymerase to bind and to initiate the polymerization of ribonucleotides. The initial characterization of eukaryotic RNA polymerase II promoters revealed the presence of a conserved sequence motif TATAAA (the TATA box) positioned 20 to 30 base-pairs (bp) upstream from the site of transcription initiation (designated +1), another conserved element GGT/CCAATCT (the CCAAT box) located around -70, as well as in some instances the GGGCGG sequence motif (the GC box) (Schibler and Sierra, 1987, Maniatis et al., 1987). These elements bind ubiquitous trans-acting factors but may also bind tissue- or developmental stage-specific effector molecules (Schibler and Sierra, 1987).

Recent studies indicate that transcription initiation of most eukaryotic genes proceeds through the ordered assembly of at least five cellular factors (TFIIA, TFIIB, TFIID, TFIIE, and TFIIF) at the highly conserved TATA box (Sawadogo and Sentenac, 1990, Lewin, 1990). These DNA-protein interactions either regulate the efficiency of transcriptional initiation or specify the transcription initiation site. One of the earliest steps in the formation of a functional preinitiation complex entails the site-specific binding of TFIID to this motif, a reaction that may be facilitated by the prior non-specific association of TFIIA to the promoter. Mutational analysis has demonstrated for many promoters that the integrity of the TATA box is essential for the maintenance of both the accuracy and efficiency of transcription initiation. Therefore, TFIID, by virtue of its specific association with this motif, represents a central target through which the action of various promoter-specific regulatory factors may be channeled. RNA polymerase II initiates mRNA synthesis upon recognition of this complex. The complex is formed and stabilized by specific interactions between the proteins and cognate DNA sequences and by protein-protein interactions between the various components. One feature of RNA polymerase II which may contribute to its interaction with the transcription complex is a conserved seven amino acid sequence that is repeated many times at the carboxy terminus of the largest subunit of the enzyme (Allison et al., 1988, Zhang and Corden, 1991). This "tail" is conserved from yeast to human and, by site-directed mutagenesis was shown to be required for transcription *in vitro* and *in vivo* (Scafe et al., 1990, Sawadogo and Sentenac, 1990).

A large subclass of RNA polymerase II promoters lacks both TATAAA and CCAAT sequence motifs but contain multiple GC boxes. This promoter class includes several housekeeping genes (dihydrofolate reductase (DHFR) (Blake et al., 1990, Means and Farnham, 1990), adenosine deaminase (Valerio et al., 1985)), as well as nonhousekeeping genes such as the mouse/rat tissue-type plasminogen activator (Feng et al., 1990), synapsin I (Sauerwald et al., 1990), epidermal growth factor receptor (Ishii et al., 1985), nerve growth factor receptor (Sehgal et al., 1988) and human transforming growth factor α (Jakobovits et al., 1988) genes. A nucleotide motif has recently been identified in the murine TATAless DHFR promoter which functions specifically to position the site of transcription initiation from several housekeeping genes (Means and Farnham, 1990). This initiator element was shown to be distinct from the TATA box. Supporting this result, a more recent report demonstrated, in the hamster DHFR promoter, that the interactions at the GC boxes were shown to be required not only for efficient transcription but also for regulating start site utilization (Blake et al., 1990). These results suggest that alternative mechanisms of transcription initiation exist, one TATA box-dependent and the other TATA box-independent. TATA box-dependent and -independent transcription initiation complexes may, by virtue of distinct factor composition and surface topography, represent differential targets for the action of various trans-acting regulatory factors.

1.2 Enhancers and modulation of transcription

Enhancers are transcriptional control elements that can modulate transcription of genes (reviewed in Maniatis et al., 1987 and Atchison, 1988). Enhancers have been located within the 5'- and 3'-flanking regions of genes as well as within introns in some instances (Queen and Stafford, 1984, Horton et al., 1987, Rossi and Crombrugghe, 1987, Bornstein et al., 1987). The properties of enhancers include their ability to 1) increase transcription of cis-linked promoters, 2) operate in an orientation- independent manner, 3) exert an effect over large distances independent of position and 4) enhance the expression of heterologous promoters.

As transcriptional control sequences have been studied in greater detail, the distinction between promoter and enhancer elements has become less clear. DNA segments that were originally thought to be promoter elements were found to show enhancer activity, and certain enhancer motifs behaved as promoter elements (Lennon and Perry, 1985). The detailed characterization of DNA sequences exhibiting enhancer activity led to the concept that an "enhancer effect" is produced by the combination of various sequence motifs, or modules, each contributing to the overall activity of the enhancer (Serfling et al., 1985). This modular arrangement of DNA segments is nicely illustrated by the upstream regions of the mouse and human metallothionein genes. These transcriptional control regions contain distinct modules that are responsive to transcriptional induction by phorbol esters, heavy metals or glucocorticoids (Serfling et al., 1985). The concept of the jigsaw puzzle was then introduced by Yamamoto et al. (1985) as an analogy to the enhancers: a regulatory protein that is to fit into an enhancer must be compatible with multiple surfaces: these surfaces include both its cognate DNA sequence motif and protein structural motifs presented by adjacently bound proteins. In other words, in order for a protein to participate in the formation of an active enhancer complex, it must be suited for specific interactions not only with a cis-regulatory DNA sequence within the enhancer, but also with protein interfaces contributed by factors localized at adjacent positions within the enhancer region.

Some of the enhancer motifs required for transcriptional induction by various agents include the glucocorticoid response element (GRE), which binds the glucocorticoid receptor, the heavy metal responsive elements of the metallothionein genes, the serum responsive element of the c-fos gene, the heat shock response elements of the heat shock genes and the phorbol ester-inducible element that binds the AP1 factor (reviewed in Johnson and McKnight, 1989). The enhancer sequence motifs have generally been found as binding sites for one or more trans-acting nuclear factors. Thus, the enhancer effect depends not only upon the mixture of sequence motifs comprising a particular enhancer region, but also upon the assortment of trans-acting factors present within a particular cell type. This second level of complexity is particularly important because a number of enhancer motifs bind to more than one trans-acting factor (as seen later), some of which are restricted in their tissue distribution. These apparently tissue-specific factors are sometimes present in many tissues in an inactive or masked form that can be converted to an active form by a post-translational mechanism (as discussed later). The ability to induce this conversion by various agents has important consequences for the tissue-specific and developmental control of transcription. Similarly, the inclusion of negative-acting motifs in some transcriptional control regions contributes to the restricted expression of some genes in certain cell types (as seen later). In support of the composite characteristic of enhancers, several reports showed that many of the basic enhancer motifs identified in specific gene enhancers were also present in other cellular enhancers (Lenardo and Baltimore, 1989, Jones et al., 1988).

As more genes were examined and more enhancer sequences identified, it became clear that in order to understand how enhancers function, one had to obtain information on the factors capable of binding to them. So, the race was on to identify cognate DNA-binding proteins. The identification was mostly done using gel retardation and competitive assays. Then, through direct screening of expression libraries with radiolabeled DNA ligands (defined during the first series of experiments), the genes encoding these proteins were cloned, sequenced and, in some cases further characterized by X-ray crystallography.

The past five years have provided a large amount of information on regulatory proteins (Harrison and Aggarwal, 1990, Landschulz et al., 1988, Johnson and McKnight, 1989, Churchill and Travers, 1991). It was shown that proteins recognize DNA in much the same way that they recognize other proteins. That is, they first form a tertiary shape or contour that is compatible with the surface with which they must interact. As in the interaction between two proteins, the atomic contacts between protein and DNA are varied, including hydrogen bonding, ionic interactions and hydrophobic interactions. It was also discovered that many different proteins recognize DNA by virtue of common structural motifs. To date, four different structural motifs have been identified: 1) helix-turn-helix (Harrison and Aggarwal, 1990, Johnson and McKnight, 1989), 2) zinc finger (Berg, 1990, Vallee et al., 1991), 3) leucine zipper (Landschulz et al., 1988, Vinson et al., 1989, Johnson and McKnight, 1989), and 4) helix-loop-helix (Murre et al., 1989, Johnson and McKnight, 1989).

As more genes encoding DNA-binding proteins are cloned using direct screening of expression libraries with radiolabeled DNA ligands and characterized, it is likely that more structural DNA-binding motifs will be discovered. These as yet unidentified motifs and the observations that some proteins contain more than one motif will add more complexity to the already sophisticated DNA-protein and protein-protein interactions.

Although the past five years have shed some light on the intricate mechanisms of control of gene expression in eukaryotes, they have at the same time generated a considerable amount of information that has yet to fall into place. The old concept that one specific protein would bind solely to its cognate DNA sequence has been proven wrong numerous times. Many laboratories have provided evidence that purified proteins have the capacity to bind with equal avidity to sites on DNA that share only minimal nucleotide sequence similarity. This represents the case where the same protein binds to multiple DNA sequences. Such is the case for the glucocorticoid receptor protein which can bind to the degenerate octanucleotide AGAa/tCAGa/t (Payvar et al., 1983, Renkawitz et al., 1984) and for C/EBP, a rat liver protein which can bind to a degenerate nonamer sequence TGTGGa/ta/ta/tG common to many animal virus enhancers (Weiher et al., 1983) as well as to the CCAAT box of eukaryotic genes (Costa et al., 1988). Since C/EBP bound avidly to both CCAAT homologies and enhancers, it was eventually termed CCAAT/enhancer binding protein (C/EBP). Homeodomain proteins are each capable of binding to sets of DNA sequences whose members diverge considerably from a consensus (Beachy et al., 1988, Desplan et al., 1988).

These observations led to the hypothesis that a DNA-binding protein which exhibits promiscuous interaction specificity *in vitro* would acquire substantially improved specificity when complexed with one or more other proteins *in vivo* (Struhl, 1989, Johnson and McKnight, 1989) It was further proposed that an auxiliary factor which improves the binding specificity of a gene regulatory protein might operate in two ways. It could contribute, from its own polypeptide structure additional contact surfaces with DNA. Alternatively, the role of an ancillary protein could be indirect. Its contacts with a second regulatory protein could induce an allosteric change in the protein, causing it to bind certain DNA sequences more avidly.

In addition to the unanticipated flexibility of binding specificity exhibited by certain regulatory proteins, recent examples where unique DNA sequence elements are capable of binding multiple regulatory proteins have also been seen (Riggs et al., 1991, Andrisani et al., 1988). This phenomenon has emerged from the study of immunoglobulin genes and studies of proteins that bind to the CCAAT elements and cAMP response element. A conserved sequence, usually referred to as the octamer element (ATGCAAAT) was found within the Ig heavy chain enhancer and promoter, and within the Ig light chain promoter (Schaffner, 1989). This octamer was found to be an important determinant of lymphoid cell-specific transcription. However, it was also found within genes whose expression is not restricted to lymphoid cells. Examples of these include histone H2b, herpesvirus tk, U1 and U2 snRNAs, enhancers of SV40 and herpesvirus immediate early genes. Numerous studies have supported the notion of multiple octamer-binding proteins (Wirth et al., 1991, Schaffner, 1989, Johnson and McKnight, 1989). Following gel retardation assays, the lymphoid octanucleotide activity has been termed OCT2 whereas the ubiquitous protein has been designated OCT1. Comparison of the protein sequence of OCT1 and OCT2 revealed that, while both proteins exhibit a helix-turn-helix motif for DNA recognition, a leucine repeat was present only in OCT2 (outside the region thought to be necessary for DNA recognition). It was suggested that this leucine repeat motif may contribute to the specificity of action of OCT2.

The CCAAT sequence element is commonly found 50 to 100 bp upstream from the transcription start site of eukaryotic genes and is important in promoter function (McKnight and Tjian, 1986). Many different CCAAT-binding proteins exist and have been detected in extracts of a variety of cell types (Dorn et al., 1987, Chodosh et al., 1988, Maity et al., 1988). There is no "universal" CCAAT-binding protein. Each protein binds to a subset of CCAAT boxes, and these specificities form partially overlapping sets. It has been shown that multiple CCAAT-binding proteins can exist within the same cell. A similar situation has been observed in the case of cAMP consensus sequence, TGACGTCA, binding site for the CREB protein. This nucleotide motif is highly homologous to another sequence TGACATCA which binds AP1 factors. Evidence has been given that the CREB protein recognition sequence can interact with AP1 and that the reverse is also possible (Andrisani et al., 1988).

Another feature which appears to be common among DNA-binding proteins is their heterodimeric structure. It is generally assumed that a given DNA-binding protein is fully capable of interacting with its target site. However, there are instances in which two proteins together can bind DNA whereas neither protein can bind alone. Examples for binding specificities that consist of multiple polypeptides include the CCAAT-binding proteins (Chodosh et al., 1988, Umek et al., 1991), the yeast mating-type regulatory proteins (Bender and Sprague, 1987) and the fos/jun proteins (Kouzarides and Ziff, 1988, Ransone et al., 1990). The combinatorial DNA-binding might increase the precision of transcriptional regulation by modulating gene expression only when two specific physiological conditions occur. It might also increase the flexibility of regulation by having a given protein associate with a variety of different proteins to yield heterodimeric species with distinct sequence recognition properties.

In some other instances, two proteins are necessary for activation even though each protein can bind independently and simultaneously to the target (Widom et al., 1991, Comb et al., 1988, Bowlus et al., 1991, Andersen et al., 1990). In such cases as well, synergy between activators may increase both the precision and flexibility of transcriptional regulation. Synergy is believed to minimize the number of distinct transcription factors that are necessary to achieve the wide variety of regulatory responses. This phenomenon has been observed with the yeast mating-type regulatory proteins.

1.3 Silencers and modulation of transcription

By virtue of the way in which the cis-acting elements have been analysed, most of the data for a long while reflected the nature of sequences needed to give expression under a given set of circumstances, i.e. positively-acting elements. More recently however, it has become apparent that sequence-specific negative regulation is a common place event in eukaryotic transcriptional control (Goodbourn, 1990, Atchison, 1988, Struhl, 1989). Some of the earliest evidence for the existence of trans-acting repressor molecules came from experiments which demonstrated that inhibitors of protein synthesis could induce specific gene expression. Derepression of gene expression has been observed for β-interferon (Ringold et al., 1984), c-myc (Greenberg et al., 1986), c-fos (Sassone-Corsi and Verma, 1987), IL2 (Efrat and Kaempfer, 1984), and platelet-derived growth factor responsive genes (Hall et al., 1989).

Another line of evidence for specific trans-acting repressor molecules came from cis-acting sequence mapping approaches. In the same way that deletion of key sequences with a corresponding loss of expression implies loss of positively-acting elements, an increase in expression is taken as evidence for deletion of a negatively-acting element. Using this approach, negative elements have now been identified in a large number of transcriptional control elements including those within the mouse Ig heavy (H) chain enhancer (Kadesch et al., 1986) and the genes encoding rat glutathione transferase P (Imagawa et al., 1991), chicken lysozyme (Baniahmad et al., 1987), mouse α-fetoprotein (Hammer et al., 1987, Vacher and Tilghman, 1990), rat collagen II (Savagner et al., 1990), mouse N-ras (Paciucci and Pellicer, 1991), chicken cardiac myosin light chain 2 (Shen et al., 1991), mouse TCR α/β (Winoto and Baltimore, 1989a), mouse γF-crystallin (Liu et al., 1991) and the yeast mating-type genes (Brand et al., 1985).

Some of the identified negative elements have been shown to have properties of silencers (negative enhancers) and thus exert their effects relatively independent of position (TCR α/β, collagen II, rat glutathione, yeast silencers); while others have more stringent position requirements (α-fetoprotein). Some negative elements appear to be promoter-specific (γF-crystallin) while some worked well on heterologous promoters (those of collagen II, N-ras, TCR α/β, IgH chain). Vacher and Tilghman (1990) have recently described the first mammalian repressor to have functional significance *in vivo* using transgenic mice, thus emphasizing the importance of such negative-acting sequences in gene regulation.

One feature shown to be common to a number of genes with negative elements is that the promoters themselves are not cell-type specific (Goodbourn, 1990). Instead, adjacent negative-acting elements repress expression in inappropriate cell types. These results emphasize the importance of concerted action of multiple regulatory elements. In agreement with the complexity of the phenomenon of gene repression, several instances have been described where the same DNA sequence was able to mediate either negative or positive transcriptional effects. Examples of such dual function include the IgH chain enhancer region (Wang et al., 1991, Lenardo et al., 1989), the serum response element (inner core) of c-fos (Rivera et al., 1990), and the regulatory sequences of the mouse proliferin gene (Diamond et al., 1990). Examples also exist where a single protein can serve either as a transcriptional repressor or activator depending on the promoter (Pei and Shih, 1991, Stenlund and Botchan, 1990). Lamph et al. (1990) recently demonstrated the dual function of the cAMP responsive element binding protein (CREB). In the absence of phosphorylation, CREB was shown to function as a repressor. However, following phosphorylation by protein kinase A, CREB exhibited a positive role (Yamamoto et al., 1988, Gonzalez and Montminy, 1989). There is now evidence to suggest that this phosphorylation of CREB allows allosteric activation of a domain capable of transcription transactivation (Gonzalez et al., 1991). It would appear that CREB can bind to promoters containing CRE-like sites but acts as a repressor until protein kinase A phosphorylates the CREB, at which time it becomes a transcriptional activator. In contrast, the erbA and jun proteins have been shown to behave in a reverse manner, i.e. after phosphorylation the protein acts as a repressor (Glineur et al., 1990, Boyle et al., 1991). In this regard, the regulation of the activity of protein kinase A represents a key event in controlling the activation of these transcriptional factors which themselves regulate specific genes.

Transactivation of factors through phosphorylation and dephosphorylation has gained a lot of importance recently (Berk, 1989, Jackson et al., 1990, Merino et al., 1989, Denner et al., 1990, Boyle et al., 1991). Evidence now exists that other posttranslational modifications such as myristylation occur (Kamata et al., 1991). There are also instances where a non-cell-type-specific protein can act as a corepressor or coactivator depending on the cell type-specific protein with which it acts in combination (E2 of bovine papilloma virus type 1 (Stenlund and Botchan, 1990) and the fos/jun proteins (Rivera et al., 1990)). Interactions with multiple auxiliary proteins may be required to bring about the positive or negative activity of that protein.

To date, four basic models have been presented to explain the various types of repression found in eukaryotes: 1) repression by steric occlusion i.e. a direct competition for overlapping sites between positive and negative transcription factors (seen in regulation of the α1 collagen gene (Karsenty and de Crombrugghe, 1990), β-interferon (Goodbourn and Maniatis, 1988), Igκ (Pongubala and Atchison, 1991), CREB (Deutsch et al., 1988, Goodbourn, 1990) and regulation by glucocorticoids (Akerblom et al., 1988)), 2) factors that stimulate transcription are held in an inactive form by protein-protein interaction with repressing factors (such as in the control of cell-type specific genes in yeast (Goodbourn, 1990)), 3) a positively-acting factor may be bound to DNA but have its transcriptional activating potential masked by association with an inhibitory factor (observed in the repression of galactose expression in yeast [gal4/gal80 interaction] (Goodbourn, 1990, Johnson and McKnight, 1989)), and 4) an inhibitor prevents the association of activator with DNA (IκB/NFκB interaction (Lenardo and Baltimore, 1989, Ghosh and Baltimore, 1990, Urban and Baeuerle, 1990), heat shock transcription factor (Berk, 1989), CREB (Berk, 1989), glucocorticoid hormone receptor (Johnson and McKnight, 1989)).

1.4 "Local" versus "general" regulation

Another difficult task linked to the study of transcriptional regulation in eukaryotes is the integration of these concepts of "local" regulation at the level of chromatin. Indeed, transcriptional regulation occurs in the context of genomic DNA packaged into multiple levels of chromatin structure (Kamakaka and Thomas, 1990, Mirkovitch et al., 1984, Morse et al., 1988). Any change in this conformation will have a direct impact on the transcriptional activity of a gene (Almouzni et al., 1991). Numerous studies have demonstrated that sequences immediately upstream from transcribed genes have an enhanced accessibility to a wide variety of nucleases such as DNAse1 (reviewed in Gross and Garrard, 1988, Elgin, 1988). Recent reports suggested that hypersensitivity reflects the local displacement or modifications of nucleosomes over promoter and promoter-proximal regions by sequence-specific transcription factors (Fascher et al., 1990, Bergman, 1986). In some instances, however, DNAse1 hypersensitive regions extend over several kilobases (kb) of DNA and suggest a structural modification of a whole chromosomal domain (Gross and Garrard, 1988, Elgin, 1988). This relaxation of higher levels of chromatin is seen as an obligatory step before the onset of transcription. Likewise, repression appears to be a consequence of the exclusion of the transcriptional machinery from regulatory regions following a change in the higher order structure of the nucleosomes (Goodbourn 1990, Mandal et al., 1990).

It has been shown that genomes are compartmentalized into active and inactive domains. Specific organiser sequences designated SARs for scaffold attachment regions have been identified recently (Mirkovitch et al., 1984, Goodbourn, 1990, Adachi et al., 1989). As their name implies, they organize chromosomal DNA into loops of 30-200 kbp and prevent the spread of the inactive or active domains in the genome. The loops are attached via sequence-specific DNA-protein interactions to a scaffold structure that lies along the chromosomal axis (von Kries et al., 1991). SARs appear to be evolutionarily conserved and are composed of approximately 250 bp DNA sequences rich in A and T residues. These sequences contain consensus cleavage sites for topoisomerase II, which is a major component of the chromosome scaffold (Spitzner et al., 1989). SARs have been identified in a variety of genes and appear to map to DNA segments lying very near enhancer or silencer regions (Cockerill and Garrard, 1986, Cockerill et al., 1987, Gasser and Laemmli, 1986, Winoto and Baltimore, 1989a). It has been suggested that SARs may bring enhancer- or silencer-containing genes into a nuclear compartment rich in transcription factors (Mukherjee et al., 1988, Hofmann et al., 1989). Based on this concept, it was suggested that genes are turned on or off by creating an environment of active or inactive chromatin in their immediate vicinity.

1.4.1 Sodium butyrate as a modulator of the chromatin structure

Sodium butyrate has been shown to modulate the structure of chromatin in many cell types and to influence the activity of gene loci contained in the targeted regions. An overview of its effects and mode of action are discussed in this last portion of this section since Chapter IV will address this issue.

Direct evidence exists for an involvement of sodium butyrate in alteration of chromatin structure. Early studies have demonstrated that the effect of butyrate is to inhibit histone deacetylation both *in vitro* and *in vivo* (Boffa et al., 1978; Candido et al., 1978; Sealy and Chalkley, 1978). An interesting study by Bode et al. (1983) using nucleosomal core particles from sodium butyrate-treated cells has revealed that hyperacetylation appear to provide particles with a considerable conformational freedom. Similar results have been obtained very recently by Oliva et al. (1990) using electron spectroscopic imaging to study the structure of the nucleosomes. They have shown that hyperacetylation of histone H4 induced by *in vivo* treatment of HeLa cells in culture with butyrate correlates with the adoption of an elongated or unfolded shape by the core particle of nucleosomes. It has been suggested that histone acetylation could change intranucleosomal interactions and thus provide or remove sites for the binding of histone H1 or other more specific DNA-binding factors. In other words, acetylation may cause the production of "waves" of changing chromatin structure which in turn could rapidly and reversibly expose sites in chromatin domains to DNA binding proteins. It is well known that alterations of histone structure have considerable relevance to the organization of chromatin and thereof to the control of gene expression and cell growth (Weintraub, 1984; Reeves, 1984; Ridsdale et al., 1990).

Butyrate may differentially modify the chromatin of different genes and prevent them from responding similarly to a common inducer. This suggestion has been given by Birren and Herschman (1986) to explain why butyrate has distinctly different consequences, in the same cell, on the dexamethasone induction of two specific mRNA.

Butyrate does not block dexamethasone induction of metallothionein (MT)-I RNA accumulation in rat hepatoma cells but does prevent induction of tyrosine aminotransferase (TAT) message by dexamethasone. The 11 fold increase hormone-induction in TAT RNA is reduced to a 2 fold increase, 8 hours after butyrate addition. Induction by glucocorticoid hormones may involve different pathways for different genes, with butyrate-sensitive steps required in some cases. More recently, Bresnick et al. (1990) have provided compelling evidence that the negative effect of butyrate on glucocorticoid responsiveness of chromatin templates is due to a modification of the chromatin that prevents promoter activation rather than due to the inactivation of a factor that is required for transactivation of the MMTV promoter under study. Their experiments have clearly demonstrated that the nucleosome is not only a means by which DNA is packaged, but is also an important component of eukaryotic gene regulation.

Table I.3 lists examples of genes examined following sodium butyrate treatment of a variety of cell lines. While some genes are strongly stimulated by this agent (c-fos (20 fold), c-sis (15 fold) [Tang et al., 1990], multidrug resistance (mdr)-1/P-glycoprotein (25 fold) [Mickley et al., 1989], glucose-regulated proteins (GRP) 78 (80 fold) and 94 (10 fold), Factor VIII (19 fold) [Dorner et al., 1989], adenine phosphoribosyltransferase (APRT) (38 fold) [Tang and Taylor, 1990]) others are repressed (CDC2, c-myc [Charollais et al., 1990], thyroid receptor 2, growth hormone [Lazar, 1990]) or are indifferent to the treatment (ras [Tang et al., 1990], β-actin [Lazar, 1990; Rius et al., 1990], α-tubulin [Charollais et al., 1990]). In addition, among the genes that show sensitivity to butyrate, there are considerable variability in the rate of modulation (fos (20 fold after 30 min.) versus sis (15 fold after 96 hours) [Tang et al., 1990], mdr-1/P-glycoprotein (25 fold after 24 hours) versus TGFα (10 fold after 48 hours) [Mickley et al., 1989]). In some instances, changes in specific gene expression reflect an increase or decrease in the rate of transcription concomittantly with an increase or decrease in the level of protein (metallothionein-1 [Birren and Herschman, 1986], cellular retinoic acid binding protein

TABLE I.3 Modulatory effects of sodium butyrate on endogenous and exogenous genes in a variety of cell types/lines. [a]In cases where no specific fold is indicated, the authors of the respective studies did not perform densitometry analysis to quantitate the modulation of mRNA and/or protein levels following sodium butyrate treatment. [b]Abbreviations are: multidrug-1/P-glycoprotein (mdr-1/Pgp), transforming growth factor-α (TGF-α), thyroid receptor β1 or α1 (TRβ1, TRα1), pregnancy-specific β1 glycoprotein (PSβ1G), cellular retinoic acid binding protein (cRABP), adenosine phosphoribosyltransferase (APRT), glucose-regulated proteins (GRP) 78 and 94, dihydrofolate reductase (DHFR), nerve growth factor (NGF).

Cell types/lines	[sodium butyrate] / length of treatment	mRNA or protein fold induction or repression	Reference
Rat glioma cells	2.5 mM, 1 to 4 days	↑ fibronectin mRNA[a] ↑ collagen mRNA ↑ sis mRNA, 5x (24 hrs) 15x (96 hrs) ↑ fos mRNA, 20x (30 min. up to 2 hrs) no change in Ki-ras mRNA	Tang et al. (1990)
Human colon carcinoma cells	2.0 mM, 6 hrs to 7 day	↑ mdr-1/Pgp mRNA/protein, 20-25x (24 hrs) ↑ TGFα mRNA, (only after 2 days)[b]	Mickley et al. (1989)
Rat pituitary adenoma cells	10.0 mM, up to 24 hrs	↓ rat growth hormone mRNA, (50%, 6 hrs) ↓ thyroid receptor β2, (50%, after 2 hrs) no change in TRβ1, TRα1 mRNA no change in β-actin mRNA no change in c-erbAα2 mRNA	Lazar (1990)
Human placental fibroblasts	1.0 mM, up to 4 days	↑ PSβ1G mRNA/protein, 8-12x	Chou et al. (1990)
Human foreskin keratinocytes	1.0 mM, 4 days	↑ plasma membrane transglutaminase ↑ cRABP protein, 7x	Schmidt et al. (1989)
Swiss 3T3 cells	5.0 mM, 1 to 7 days	↑ c-fos mRNA ↑ aP2 (adipocyte P2) mRNA ↑ lipoprotein lipase mRNA ↑ adipsin mRNA	Toscani et al. (1990)
Mouse erythro-leukemic cells	5.0 mM, up to 5 days	↑ α- and β-globin mRNA/protein	Beru et al. (1990)
Mouse 3T3 fibroblasts	6.0 mM, 12 hrs	↓ c-myc mRNA ↓ cdc2 mRNA no change in α-tubulin mRNA no change in c-jun mRNA	Charollais et al. (1990)
Mouse L cells	5.0 mM, 2 days	↑ APRT protein, 8-38x	Tang and Taylor (1990)
CHO cells	5.0 mM, 24 hrs	↑ erythropoietin mRNA, 6x ↑ Factor VIII mRNA, 10-19x ↑ von Willebrand factor mRNA, 8x ↑ adenosine deaminase mRNA, 8x ↑ GRP-78 mRNA, 50-80x, GRP-94, 8-10X no change in DHFR mRNA	Dorner et al. (1989)
Human promonocytic leukemic cells	1.0 mM, 1 to 5 days	↑ vimentin mRNA no change in β-actin mRNA	Rius et al. (1990)
Rat pheochromocytoma cells	6.0 mM, 30 min. to 24hrs	↑ preproenkephalin mRNA/protein ↑ enolase mRNA/protein ↑ fos mRNA/protein, 6x (after 30 min.) ↑ jun mRNA/protein, 6-8x (after 6 hrs) ↓ tyrosine hydroxylase mRNA/protein ↓ NGF receptor mRNA	Naranjo et al. (1990)

(cRABP) [Schmidt et al., 1989], γ-globin [Perrine et al., 1989], mdr-1/P-glycoprotein [Mickley et al., 1989]). In other cases, posttranscriptional processes are affected (fos, sis [Tang et al., 1990], plasma membrane transglutaminase [Schmidt et al., 1989], APRT [Tang and Taylor, 1990]).

Table I.3 shows that butyrate has distinctly different consequences in the same cell on the induction of specific RNAs. Two models have been put forward to explain such differences between sensitivity of genes within the same cell. As briefly mentioned above, the first hypothesizes that butyrate facilitates gene expression by preventing histone deacetylation. This would freeze the chromatin in an "open" conformation and thus allow RNA polymerase II complexes to read through continuously (reviewed in Kruh, 1982). An alternative model implicates post-translational modifications of cellular DNA-binding proteins. This could result in alterations of the network of interactions between transcriptional factors that bind to regulatory regions and consequently change the fate of gene expression (Partington et al., 1984, Naranjo et al., 1990, Stoddart et al., 1989). Although direct evidence for such *de novo* modifications of transcriptional factors has not been published yet, nuclear proteins such as histones and non-histone high-mobility-group (HMG) proteins have been modified following butyrate addition (Boffa et al., 1981, Levy-Wilson, 1981).

Clearly, regulation of eukaryotic gene expression at the transcriptional level is a very complex phenomenon, much more sophisticated than was first anticipated. Most cis-acting regulatory sequences, such as tissue-specific promoters, enhancers, and silencers are composed of many individual target elements and hence interact with the products of multiple trans-acting loci. It is the combinatorial effect of this multitude of factors that results in the significant up or down regulation of gene expression. This aspect of eukaryotic gene regulation renders the analysis of expression of any gene more difficult since it implies that the behaviour of any given regulatory element will depend upon the context in which it is found. The balance between silencer or enhancer accessible binding sites and available transcriptional factors will undoubtedly be a key element in the understanding of tissue-specific expression.

2. *Regulation of gene expression in T cells*

From the moment T cell precursors reach the thymus, they acquire many different T cell surface molecules that will be crucial differentiating elements. A myriad of genes are sequentially activated and specifically regulated during this process to allow for proper homing of the various T cells to their specific locations and prepare T cells for their eventual encounter with the antigens. Most of these genes are lineage-specific and are expressed at very specific times during either T cell development or T cell activation. As described in this section, transcription of most if not all these genes is controlled by the activities of nuclear regulatory proteins that are modulated during these processes.

2.1 Gene regulation during T cell differentiation

2.1.1 CD3 genes

The earliest definitive signal of commitment to the T cell lineage is expression of the CD3 genes (von Boehmer, 1988). The coordinate transcription of the CD3 chain genes precedes TCR rearrangement and may even occur prior to migration to the thymus (Haynes et al., 1989). The intracellular expressed CD3 γ, δ, ε, ζ proteins form a subcomplex to which the TCR α and β chains bind noncovalently before the complex can be transported to the cell surface (Terhorst et al., 1988, Davis, 1990). The delineation of the regulatory elements responsible for the exclusive expression of the CD3 genes in T cells has been achieved recently following successful cloning and sequencing of both cDNA and genomic copies of some of the individual chains.

One interesting aspect of the CD3 genes analysed so far is that none of the CD3 promoters contain a TATA box or a CCAAT box or GC boxes (Tunnacliffe et al., 1986, Terhorst et al., 1988). Site-directed mutagenesis experiments are underway to define the motifs that promote initiation of transcription. Further work is required to determine if silencers are responsible for repressing the CD3 genes in non-T cells during development.

2.1.1.1 CD3 δ

A transcriptional enhancer region (approximatively 400 bp in length) was identified 600 bp downstream of the polyadenylation signal. This enhancer was found to be active only in cells of the T lineage; however, the promoter of CD3 δ revealed no tissue-specificity suggesting that the enhancer located 3' of the gene is the major tissue-specific element (Georgopoulos et al., 1988). In a second report, Georgopoulos et al. (1990) identified two cis-acting elements designated δA and δB within the 400 bp enhancer region responsible for mediating the enhancer function. Element δA is the major element responsible for mediating changes in CD3 δ gene expression during T cell development. δB is however required for full activity of the enhancer. Elements similar to δA have been found in the TCR α chain enhancer, where deletion of this sequence destroyed enhancer activity, and also in the promoter regions of the various TCR β chain genes. Sequences homologous to the δB element have also been detected in the TCR β chain enhancer and in the IL2 promoter revealing the composite aspect of enhancers. The way in which δB-binding activity amplifies the δA factor-activating potential is unclear at this point, although it has been suggested that posttranscriptional modifications taking place during T cell development may confer higher affinity and activity to the δA factor.

2.1.1.2 CD3 ε

As is the case with the other CD3 genes, the CD3 ε gene is expressed uniquely in all cells of the T lymphocyte lineage. A T cell-specific enhancer (320 bp) was found 12 kb downstream of the last exon and coincided with a CpG island and a T cell- specific DNAse1 hypersensitive site (Clevers et al., 1989). The CD3 ε enhancer could be divided into three functional domains: the most 3' 125 bp were indispensable for activity and functioned as a 5 to 10 fold T cell-specific enhancer; two domains were located 5' to this core, each acted as a two fold "amplifier", but by themselves were inactive.

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2.1.2 CD2 gene

The cell surface glycoprotein CD2 is found exclusively on T lymphocytes. Its presence is first detectable shortly after the entry of progenitor cells into the thymus and thereafter it is found on over 95% of thymocytes and on all peripheral T cells (Terhorst et al., 1988, Davis, 1990). Lake et al. (1990) have identified an enhancer region (550 bp in length) in the 3'-flanking sequences that maps to the dominant control region (DCR) of the CD2 gene and consists of six cis-acting sequence motifs. A footprint over one element defines a canonical cAMP responsive element (CRE), a target for a binding protein induced by increased levels of intracellular cAMP (CREB). Lake's report has also indicated that in a transient assay, the CD2 enhancer fused to the herpes virus thymidine kinase promoter is not functional in HeLa cells but is active at a low level in B cells. This result demonstrates that the enhancer is probably not the complete mediator of tissue-specific gene expression and that absolute specificity requires the presence of the CD2 promoter sequences. Another suggestion that has been given is that the region of chromatin in which the CD2 gene is situated contributes to the tissue-specificity of expression. These conclusions are consistent with and complementary to the finding that a 5.5 kb fragment at the 3'-end of the human CD2 gene (28 kb) confers high level, position-independent gene expression in transgenic mice (Greaves et al., 1989). The CD2 3'-flanking region and more specifically the DCR contains both topoisomerase II and matrix attachment region consensus sequences as well as an A-T rich region characteristic of scaffold attachment. These have been involved in chromatin structure and assembly and have been strongly implicated in the formation of active chromatin (Adachi et al., 1989, Spitzner et al., 1989). It is possible that the activity that defines the DCR is an intrinsic property of the CD2 enhancer.

2.1.3 T cell receptor genes

The predominant T cell form that develops in the thymus carries an antigen receptor composed of α and β chains. A minor population of mature T cells, constituting 1-5% of those in the periphery, express distinct heterodimeric γδ TCRs. The control of TCR gene rearrangement and expression is particularly perplexing because a single locus contains the genes for both the TCR α and δ chains (Davis, 1988, 1990). During T cell ontogeny, the γ and δ TCR genes are rearranged and expressed first. T cells expressing the αβ receptors appear later. An intricate developmental control must exist in the α/δ locus so that the two genes can be expressed in different sets of T cells and be activated at different times during T cell development.

2.1.3.1 TCR α

Expression of the human and murine TCR α genes is regulated by very similar enhancers (230 bp in length) located 4.5 kb 3' to the constant gene segments (Winoto and Baltimore, 1989b). The human TCR α enhancer is required for high level transcription from α TCR promoter and is active only in TCR α/β^{+} T cells. The core enhancer has been localized to a 116 bp fragment that contains two nuclear protein binding sites, Tα1 and Tα2, both of which are required for full enhancer activity (Ho et al., 1989). Tα1 contains a consensus cAMP response element (CRE) and binds a set of ubiquitously expressed CRE binding proteins. Tα2 on the other hand, is bound by the Ets-1 protein at a 17 bp sequence at the 3'-end of the element (Ho et al., 1990). Ets-1 is a nuclear protein encoded by the murine ets-1 gene which shows strong amino acid sequence similarity to the v-*ets* oncogene product of the avian E26 leukemia virus (Karim et al., 1990). Ets-1 is expressed preferentially in cells of the T and B lymphocyte lineages. The human TCR α enhancer is however T cell-specific. Therefore, it seems unlikely that Ets-1 alone is responsible for the T cell-specific activity of this enhancer. A recent report showed that Ets-1 binds to the Tα2 site in concert with additional transcriptional regulatory proteins (Ho et al., 1990). If the expression of the auxiliary proteins were restricted to T cells, binding of the Ets-1 protein complex might be relatively T cell-specific. This represents another example of combinatorial regulation of gene expression.

A series of experiments by Winoto and Baltimore (1989a) provided further information on the intricate controls in place to prevent TCR α chain gene expression in γδ T cells. They found that the minimal α enhancer is active *in vitro* in the γδ T cell lineage but gained αβ lineage specificity through negative cis-acting elements 3' of the Cα gene that silence the enhancer *in vivo* in γδ T cells. These sequences were shown to abolish the activity of other enhancers in other cells but allow the α enhancer to function only in the context of αβ T lineage. The sequences of both silencers revealed several regions that are homologous to the consensus scaffold binding site and a consensus topoisomerase II binding site (Spitzner et al., 1989). These sequences may restrict the genes in a chromatin conformation not favorable for transcription (as discussed previously in section C.1.4).

2.1.3.2 TCR β

An enhancer was found 7.5 kb 3' of Cβ2 that has a T cell-specificity and binds two purified IgH chain enhancer factors (McDougall et al., 1988). Further analysis performed by Diamond et al. (1989) mapped the positive regulatory sequences within a 230 bp size DNA fragment. Another study performed by Anderson et al. (1989) which focused on Vβ promoter sequences, revealed a well-conserved decanucleotide sequence (AGTGACATCA) positioned at approximately 70 bp relative to the transcription start site. This motif is required for high level expression of the Vβ gene and functions *in vitro* as a high affinity site for a well characterized transcription activator, CREB. These results have been confirmed by Ratanavongsiri et al. (1990). It has been suggested that tissue-specificity of the TCR β gene could be achieved through protein/protein interactions under the influence of the 3'-enhancer.

2.1.3.3 TCR γ

Although Cγ1 and other Cγ genes are rearranged in most peripheral αβ T cells, the corresponding transcripts are usually absent. Transcriptional regulation may play an important role in the developmental decision between αβ and γδ lineages. By analogy to the TCR α and β genes, Spencer et al. (1991) recently identified a tissue-specific transcriptional enhancer located 3 kb downstream of Cγ1. In addition, cis-acting regulatory elements flanking the enhancer have been shown to suppress γ expression in αβ T cells (Ishida et al., 1990). These elements are very similar to the cis-acting elements that prevent α gene expression in γδ T cells.

Section 2.1 points to an interesting feature of the genes that are expressed during the maturation of T cells in the thymus: the presence of the sequence TGATCATCA, binding site for the AP1 protein (jun-related family) and the CREB (cAMP responsive element binding) protein. The CD3δ, CD2, TCRα, TCRβ genes all contain this recognition block. Understanding the molecular events that induce expression of T cell-specific transcription factors such as AP1 and CREB will eventually lead to the signals that initiated the cascade of events during T cell development.

2.2 Gene regulation during T cell activation

Resting human peripheral blood T cells are metabolically quiescent. DNA synthesis is undetectable and rates of RNA and protein synthesis are low (Unanue and Benecerraf, 1984, Cohen et al., 1990). When they are activated with antigenic or mitogenic stimulation, a complex cascade of biochemical events occur that eventually results in cell proliferation and the acquisition of immunologic competence (see section A.3). More than 100 molecules are specifically regulated during this process (an overview is presented in Table I.4) (Crabtree, 1989, Ullman et al., 1990). These regulatory events begin within minutes of contact with antigen and continue for at least 10 days. The role of these secondary events in conveying a signal to the nucleus is not clear. One popular strategy taken by many investigators to answer this question is the so called "reverse" approach (Ullman et al., 1990). By understanding how gene regulatory proteins are regulated, information can be obtained on the nature of the connection between the cell membrane and the nucleus. A few of the genes activated upon antigen stimulation will be discussed in this section.

2.2.1 Interleukin-2 gene

Regulation of the IL2 gene has been the subject of intensive investigation as its expression is pivotal to T cell activation. IL2 is transcribed in T helper cells only after the cell receives two independent signals 1) specific TCR activation by antigen-presenting cells in the context of MHC and 2) binding of cytokines produced by activated macrophages in a non-MHC dependent interaction. These signals are thought to potentiate calcium and protein kinase second messenger responses. The integration of these multiple signals results in the transcriptional activation of the IL2 gene and is mediated by the binding of several factors to an enhancer between -319 and -52 relative to the start site (Fujita et al., 1986, Siebenlist et al., 1986, Ullman et al., 1990).

The binding of one factor, NFAT-1 (nuclear factor of activated T cells) to the IL2 sequences -286 to -257, is closely correlated with the activation of IL2 transcription (Crabtree, 1989, Ullman et al., 1990, Arai et al., 1990). NFAT-1 binding activity, found only in activated lymphoid cells, is strongly induced within 20 minutes after stimulation of T helper cells and precedes IL2 mRNA production by 20 minutes. Induction of NFAT-1

TABLE I.4 An overview of T lymphocyte activation molecules. The categories are based on an analogy to viral gene expression. Immediate events are independent of protein synthesis; early events require protein synthesis (data not always available) but precede cell division, and late events occur after cell division. Time refers to the earliest time that the molecule can be detected or that an increase could be measured. A/NA refers to the relative levels in activated versus nonactivated cells at peak induction. In some cases this data was not available since only fluorescent intensity was measured. Location refers to the location of the molecule within the cell: N, nuclear; C, cytoplasmic; CM, cell membrane; S, secreted. NFAT-1 and NFκB are both transcription factors required in activation of many T cell genes. SAM, S-adenosylmethionine; GM-CSF, granulocyte-macrophage colony stimulating factor; CCP1, CCP2, cytotoxic cell protease 1, 2 and their alternative protein names granzymes B, C; HF, Hanukah factor or granzyme A.

Name	Time	Location	Ratio (A/NA)
Immediate			
c-fos	15 min	N	>100
NFAT-1	20 min	N	~50
c-myc	30 min	N	20
NFκB	30 min	N	>10
Early			
γ-interferon	30 min	S	>100
Interleukin-2	45 min	S	>1000
Ornithine decarboxylase	1 hr	C	10
c-abl	1 hr	CM/C	~10
SAM decarboxylase	1 hr	C	5
Insulin receptor	1 hr	CM	3
$p56^{lck}$ lymphoid cell kinase	1 hr	CM	1/4
Transforming growth factor β	<2 hrs	S	>10
Actin	2 hrs	C	3
IL2 receptor α	2 hrs	CM	>50
IL3	1-2 hrs	S	>100
Lymphotoxin	1-3 hrs	S	>100
Cyclin	4-6 hrs	C	>10
IL4, IL5, IL6	<6 hrs	S	>100
Transferrin	6-8 hrs	S	?
Transferrin receptor	14 hrs	CM	5
c-myb	16 hrs	N	100
Glucocorticoid receptor	20 hrs	N	5-6
GM-CSF	<20 hrs	S	?
Galactosyltransferase	21 hrs	CM	12
Neuraminidase	24-48 hrs	C	5
Histone H3	24-48 hrs	N	>10
Late			
CCP1/granzyme B	2 days	?	>100
MHC class II	3-5 days	CM	>100
HF/granzyme A	<5 days	?	>100
CCP2/granzyme C	<5 days	?	>100

This table was derived from Ullman et al., 1990.

binding activity is dependent on *de novo* transcription and translation. Drugs that block the induction of NFAT-1 binding activity, such as cyclosporin A or translation inhibitors, also block IL2 transcription. Deletions in the NFAT-1 binding site that abolish NFAT-1 binding reduce reporter gene expression in transient transfection assays to 5-30% of wild type activity. Collectively, these results suggest that the gene encoding NFAT-1 is one of the first genes induced as part of a complex pathway of differentiation leading to T cell immunologic function.

Another nuclear factor (NFIL2A) was identified which binds to IL2 enhancer sequences between -93 to -63. As opposed to NFAT-1, this factor is present in nuclear extracts of both activated and resting T cells. NFIL2A protein both suppresses expression from the IL2 promoter in nonactivated T cells and activates it after cells are stimulated via the TCR. This factor has recently been demonstrated to be indistinguishable from the ubiquitous octamer binding protein OCT1 which belongs to the family of proteins containing a homeodomain. They also contain the so-called POU domain identified in a number of tissue-specific and developmentally-specific transcription factors (Ruvkun and Finney, 1991). The 20 minute delay between the appearance of NFAT-1 and the first detection of IL2 mRNA may reflect the assembly of some form of functional complex. The observation that deletion of either the NFAT-1 or the NFIL2A site abolishes most of the activity of the IL2 enhancer suggests that two sites cooperate for full activity; however, each protein binds independently.

Three other footprints were detected in nuclear extracts of activated T cells within the enhancer region and presumably identify distinct protein binding sites (-208 to -188, -185 to -177 and -154 to -145). Sequences between -185 to -177 and -154 to -145 represent two separate binding sites for an element common to many genes that are transcriptionally induced by phorbol esters, namely the TPA responsive element (TRE). This sequence element TGAGTCA is recognized by the transcription factor AP1 which is encoded by the cellular protooncogene c-jun. Deletion of the AP1 site from the IL2 enhancer reduces the response of the enhancer to PMA stimulation in a number of T cell lines tested. A functional AP1 site in the IL2 gene suggests that the control region of the gene is a mosaic of elements that responds to signals emanating from the antigen receptor and to signals initiated with the activation of protein kinase C. Thus, signals initiated through two cell membrane receptors may be integrated at the level of the responsive gene. Sequences between -208 to -188 correspond to an NFκB binding site. Deletion and mutational analysis involving this site have led to conflicting results as to its importance in IL2 enhancer function. Whether this site contributes to the PMA requirement for IL2 induction or whether other PMA responsible factors carry this function remains to be elucidated.

Analysis of deletion mutations suggests that all the protein binding sites must be occupied to allow activity of the IL2 enhancer. Thus a highly cooperative interaction must occur between the various binding proteins. One implication of the requirement for full occupancy is that signals that activate only one of the enhancer elements will not give rise to transcription of IL2. This may in part account for the requirement of two signals for T cell activation. Activation of transcription factors such as AP1 or NFκB following PMA induction does not activate IL2 transcription. Likewise, activation of NFAT-1 by triggering the antigen receptor alone does not activate IL2 transcription. However, both stimuli lead to activation of each of the essential transcription factors and presumably initiate IL2 gene transcription.

A recent report has indicated that both murine and human IL2 5'-flanking regions share 86% identity from position -580 to -1 (Novak et al., 1990). Other regulatory factors, as yet unidentified must interact with sequences upstream from -320 (which comprises the previously identified enhancer) since there must be selective pressure to keep these regions so similar. The high degree of homology in the sequences between known sites suggests that some factors may bind very close to one another or even overlap in their DNA recognition specificity. Further examination has revealed the presence of positive elements in the region between -351 to -578. These sequences were not required for promoting the expression of the murine or human IL2 gene. However, certain deletions in this portion of the gene dramatically reduce expression from the IL2 promoter even when the binding sites for NFAT-1, NFκB, NFIL2A (OCT1) and AP1 were intact. Other potential positive and negative regions have been mapped further upstream -1890 and -750. These distal regulatory elements appeared to be capable of modulating levels of expression but not of activating expression autonomously.

A very interesting aspect of the IL2 gene enhancer regulation has been revealed recently by Fraser et al. (1991) who showed that stimulation of the IL2 gene via the CD28 T cell adhesion receptor induces the formation of a different protein complex over the IL2 enhancer regions along with a distinct set of binding sites. Clearly, the signals transduced from the CD28 receptor differ from those of the TCR.

2.2.2 IL2 receptor gene

Activation of the IL2 receptor (IL2R) gene expression is also essential for commitment to cell division and immunologic function (Crabtree, 1989, Ullman et al., 1990, Waldmann, 1991). High affinity binding of IL2 to its receptor involves a trimolecular interaction among IL2 and two cell membrane-bound polypeptide chains α (p55) and β (p70-75) that constitute the complete receptor. Both IL2Rα and IL2Rβ chains are capable of binding IL2 however, the β chain is alone responsible for driving the intracellular IL2 signal transduction pathways.

High affinity IL2 receptors are rapidly induced during the course of T cell activation. Since some resting T cells constitutively express IL2Rβ chain, the transcriptional activation of IL2Rα gene is important to both the regulated display of high affinity IL2R and T cell proliferation. Cell surface expression of IL2Rα can be induced by a variety of stimuli, including antigens and the nonspecific mitogens phytohemagglutinin (PHA) and PMA. The macrophage-derived cytokine, TNFα has also been shown to induce IL2Rα gene expression (Lowenthal et al., 1989, Meyer et al., 1991). Systematic deletion and site-specific mutations of the 5'-flanking region of the IL2Rα gene have led to the identification of an important regulatory element located between nucleotides -267 and -256 which is required for the mitogenic activation of this gene (Böhnlein et al., 1988). A more recent report has shown that an NFκB factor in association with another protein as yet to be characterized bind to this DNA sequence element (Böhnlein et al., 1989). PMA alone was sufficient to activate expression of these DNA binding proteins in T cells which indicates that a phosphorylation reaction involving protein kinase C may play a role in the posttranslational regulation of these trans-acting factors.

More recently, studies performed by Pomerantz et al. (1989) have indicated that a second sequence element located approximately 10 bp 3' to the NFκB site is required for optimal induction of the IL2Rα gene. This element bears considerable homology to the consensus-binding site of the transcription factor Sp1. Competition for the nucleoprotein complex formed on the IL2Rα promoter by Sp1-binding sites from the SV40 promoter further supports the notion that a protein with a similar sequence specificity as Sp1 can bind to this sequence. The presence of these ubiquitous transcription factors highlights the diverse possibilities for gene regulation attainable by appropriate juxtaposition of binding sites for inducible and constitutive transcription factors.

Besides NFκB and Sp1-like sites, the 5'-flanking region of IL2Rα gene contains a negative regulatory element (NRE) between -471 and -317 (Smith and Greene, 1989). A nuclear factor of 50 kDa has recently been identified as the binding protein for NRE. NRE appears to require additional specific cis-acting elements to effect its negative potential. Analogous to the IL2 gene, the activation of the IL2Rα gene following various stimuli (such as TNFα or PMA) involves the induced expression of at least two distinct DNA binding proteins that interact specifically with the κB-like element present in the IL2Rα promoter. It has been shown that these proteins are regulated posttranslationally.

The isolation of cDNA clones for the IL2Rβ chain gene has just recently been achieved (Hatakeyama et al., 1989). The transcriptional regulation of this important polypeptide awaits further investigation.

Both IL2 and IL2R gene expression could be under the influence of c-fos and c-myc gene activation. Indeed, both protooncogenes are rapidly induced after interaction of the TCR with antigen. Although their expression is not T cell-specific, they could participate in the cascade of events that lead to the stimulation of transcription of the essential IL2 and IL2R genes.

2.2.3 "Early" and "late" genes

The activation of at least two "early" genes (whose transcription requires protein synthesis but precedes cell division) have been found to be contingent on prior expression of IL2. By blocking the IL2R with an antibody, it has been demonstrated that the interaction of IL2 with its receptor is required for expression of the mRNA for c-myb and the transferrin receptor (Stern and Smith, 1986, Pauza, 1987, Neckers and Cossman, 1983). It has been suggested that these two genes contain sequence elements that bind transcription factors which respond to signals transduced by the IL2R. Their 5'-flanking regions could also present IL2 responsive sequence elements.

Expression of the so called "late" genes (whose transcription requires protein synthesis and takes place after cell division) such as perforin and CCPs in CTLs also require IL2 (added exogenously in culture) to be expressed. The same activation mechanisms as above can be envisaged i.e. that these genes contain IL2 responsive elements which provide a site for the direct binding of IL2 or that the factors responsible for their activation are somehow affected by the signals that are transduced from the IL2R. These various possibilities remain to be examined.

Of the numerous regulatory mechanisms that have been identified in eukaryotes, none has been shown to affect the immediate early and early gene activation more than the transcriptional controls. In order to establish causal relations between cell membrane events and the activation of genes in the nucleus, it will be essential to understand how the proteins binding to the antigen receptor response elements are activated to initiate transcription of early genes such as IL2 and IL2R. Knowing the biochemical events involved in the activation of these proteins will provide an understanding of the pathways transmitting signals from the antigen receptor to genes responsible for T cell proliferation and the acquisition of immune function. Eventually, one will be able to establish the contingent relations among the known gene activations to determine how the process is temporally ordered and how late functions such as cytotoxicity are controlled.

D. THESIS OBJECTIVES

A lot of evidence has accumulated that implicate CTL-specific serine proteases or CCPs in cell-mediated cytolysis. The genes that encode them have been shown to be activated upon T cell stimulation both *in vitro* and *in vivo* but the control(s) behind their temporal and T cell-specific expression remain(s) unknown. While the focus of many laboratories in the past five years has been on defining the substrates of the CCPs, producing antibodies against such proteins, or looking for other members of this superfamily, the mechanism(s) of transcriptional activation of the genes that encode these specialized enzymes has been neglected. One major reason for this was the difficulty in introducing exogenous DNA molecules into CTL clones. Therefore, the first step toward the identification of the regulatory elements of CCP1 (C11) and CCP2 (B10) was to establish a reproducible and efficient transfection protocol for CTLs which comprises chapter II of this thesis. A comparison of the regulatory DNA sequence elements found within the 5'-flanking region of both C11 and B10 was then established and is presented in chapter III along with a preliminary topographical map of the 5'-end for both genes. The last chapter describes the mapping of sodium butyrate-sensitive sequences within the 5'-flanking regions of C11 and B10 genes. Thus, the work described herein centers on the comparison of the transcriptional regulation of two of the closely related serine protease genes, namely C11 and B10.

BIBLIOGRAPHY

Abraham, N. and Veillette, A. (1991). Enhancement of T-cell responsiveness by the lymphocyte-specific tyrosine protein kinase p56^{lck}. *Proceedings of the Fifth Spring Meeting of the Canadian Society for Immunology*, Château Lake Louise, Lake Louise, Alberta.

Abramson, S., Miller, R.G. and Phillips, R.A. (1977). The identification in adult bone marrow of pluripotent and restricted stem cells of the myeloid and lymphoid systems. *J. Exp. Med.* **145**:1567-1579.

Adachi, Y., Käs, E. and Laemmli, U.K. (1989). Preferential, cooperative binding of DNA topoisomerase II to scaffold-associated regions. *EMBO J.* **8**:3997-4006.

Akerblom, I.E., Slater, E.P., Beato, M., Baxter, J.D. and Mellon, P.L. (1988). Negative regulation by glucocorticoids through interference with a cAMP responsive enhancer. *Science* **241**:350-353.

Allison, L.A., Wong, J. K.-C., Fitzpatrick, D., Moyle, M. and Ingles, C.J. (1988). The C-terminal domain of the largest subunit of RNA polymerase II of *Saccharomyces cerevisiae, Drosophila melanogaster*, and mammals: a conserved structure with an essential function. *Mol. Cell. Biol.* **8**:321-329.

Almouzni, G., Méchali, M. and Wolffe, A.P. (1991). Transcription complex disruption caused by a transition in chromatin structure. *Mol. Cell. Biol.* **11**:655-665.

Andersen, B., Catignani Kennedy, G. and Nilson, J.H. (1990). A cis-acting element located between the cAMP response elements and CCAAT box augments cell-specific expression of the glycoprotein hormone α-subunit gene. *J. Biol. Chem.* **265**:21874-21880.

Anderson, S.J., Miyake, S. and Loh, D.Y. (1989). Transcription from a murine T-cell receptor V_β promoter depends on a conserved decamer motif similar to the cyclic AMP response element. *Mol. Cell. Biol.* **9**:4835-4845.

Andrisani, O.M., Pot. D.A., Zhu, Z. and Dixon, J.E. (1988). Three sequence-specific DNA-protein complexes are formed with the same promoter element essential for expression of the rat somatostatin gene. *Mol. Cell. Biol.* **8**:1947-1956.

Arai, K., Lee, F., Miyajima, A., Miyatake, S., Arai, N. and Yokota, T. (1990). Cytokines: coordinators of immune and inflammatory responses. *Ann. Rev. Biochem.* **59**:783-836.

Atchison, M.L. (1988). Enhancers: mechanisms of action and cell specificity. *Ann. Rev. Cell Biol.* **4**:127-153.

Atkinson, E.A., Gerrard, J.M., Hildes, G.E. and Greenberg, A.H. (1990). Studies of the mechanism of natural killer (NK) degranulation and cytotoxicity. *J. Leukocyte Biol.* **47**:39-48.

Baniahmad, A., Muller, M., Steiner, C., Renkawitz, R. (1987). Activity of two different silencer elements of the chicken lysozyme gene can be compensated by enhancer elements. *EMBO J.* **6**:2297-2303.

Beachy, P.A., Krasnow, M.A., Gavis, E.R., Hogness, D.S. (1988). An *ultrabithorax* protein binds sequences near its own and the *antennapedia* P1 promoters. *Cell* **55**:1069-1081.

Bender, A. and Sprague Jr, G.F. (1987). MATα1 protein, a yeast transcription activator, binds synergistically with a second protein to a set of cell-type-specific genes. *Cell* **50**:681-691.

Benfey, P.N., Yin, F.H. and Leder, P. (1987). Cloning of the mast cell protease, RMCPII. Evidence for cell-specific expression and a multigene family. *J. Biol. Chem.* **262**:5377-5384.

Bentwood, B.J. and Henson, P.M. (1980). The sequential release of granule constituents from human neutrophils. *J. Immunol.* **124**:855-862.

Berg, J.M. (1990). Zinc fingers and other metal-binding domains. *J. Biol. Chem.* **265**:6513-6516.

Bergman, L.W. (1986). A DNA fragment containing the upstream activator sequence determines nucleosome positioning of the transcriptionally repressed PHO5 gene of *Saccharomyces cerevisiae. Mol. Cell. Biol.* **6**:2298-2304.

Berk, A.J. (1989). Regulation of eukaryotic transcription factors by post-translational modification. *Biochem. Biophys. Acta* **1009**:103-109.

Berke, G. (1985). How T lymphocyte kill infected cells. *Microbiol. Sci.* **2**:44-49.

Berke, G. (1989). The cytolytic T lymphocyte and its mode of action. *Immunol. Lett.* **20**:169-178.

Berke, G. and Rosen, D. (1988). Highly lytic *in vivo* primed cytolytic T lymphocytes devoid of lytic granules and BLT-esterase activity acquire these constituents in the presence of T cell growth factors upon blast transformation *in vitro. J. Immunol.* **141**:1429-1436.

Beru, N., Maples, P.B., Hermine, O. and Goldwasser, E. (1990). Differential expression of α- and β-globin genes in erythroleukemic cell lines. *Mol. Cell. Biol.* **10**:3591-3595.

Beutler, B. and Cerami, A. (1989) The biology of cachectin/TNF-α primary mediator of the host response. *Ann. Rev. Immunol.* **7**:625-655.

Bierer, B.E., Sleckman, B.P., Ratnofsky, S.E. and Burakoff, S.J. (1989). The biologic roles of CD2, CD4, and CD8 in T-cell activation. *Ann. Rev. Immunol.* **7**:579-599.

Birren, B.W. and Herschman, H.R. (1986). Regulation of the rat metallothionein-I gene by sodium butyrate. *Nucleic Acids Res.* **14**:853-867.

Bjorkman, P.J. and Parham, P. (1990). Structure, function, and diversity of class I major histocompatibility complex molecules. *Ann. Rev. Biochem.* **59**:253-288.

Blackwell, T.K. and Alt, F.W. (1988). Immunoglobulin genes. In *Molecular Immunology.* (B.D. Hames and D.M. Glover, eds), IRL Press, Washington. pp. 1-60.

Blake, M.C., Jambou, R.C., Swick, A.G., Kahn, J.W. and Azizkhan, J.C. (1990). Transcriptional initiation is controlled by upstream GC-box interactions in a TATAA-less promoter. *Mol. Cell. Biol.* **10**:6632-6641.

Blakely, A., Gorman, K., Ostergaard, H., Svoboda, K., Liu, C.-C., Young, J.D.-E and Clark, W.R. (1987). Resistance of cloned cytotoxic T lymphocytes to cell-mediated cytotoxicity. *J. Exp. Med.* **166**:1070-1083.

Bleackley, R.C., Lobe, C.G., Duggan, B., Ehrman, N., Frégeau, C., Meier, M., Letellier, M., Havele, C., Shaw, J. and Paetkau, V. (1988). The isolation and characterization of a family of serine protease genes expressed in activated cytotoxic T lymphocytes. *Immunol. Rev.* **103**:5-19.

Bloom, E.T., Umehara, H., Bleackley, R.C., Okumura, K., Mostowski, H. and Babbitt, J.T. (1990). Age-related decrement in cytotoxic T lymphocyte (CTL) activity is associated with decreased levels of mRNA encoded by two CTL-associated serine esterase genes and the perforin gene in mice. *Eur. J. Immunol.* **20**:2309-2316.

Bode, J., Gomez-Lira, M. and Schroter, H. (1983). Nucleosomal particles open as the histone core becomes hyperacetylated. Eur. J. Biochem. **130**:437-445.

Boffa, L.C., Gruss, R.J. and Allfrey, V.G. (1981). Manifold effects of sodium butyrate on nuclear function. *J. Biol. Chem.* **256**:9612-9621.

Boffa, L.C., Vidali, G., Mann, R.S. and Allfrey, V.G. (1978). Suppression of histone deacetylation *in vivo* and *in vitro* by sodium butyrate. *J. Biol. Chem.* **253**:3364-3366.

Böhnlein, E., Ballard, D.W., Bogerd, H., Peffer, N.J., Lowenthal, J.W. and Greene, W.C. (1989). Induction of interleukin-2 receptor-α gene expression is regulated by post-translational activation of κB specific DNA binding proteins. *J. Biol. Chem.* **264**:8475-8478.

Böhnlein, E., Lowenthal, J.W., Siekevitz, M., Ballard, D.W., Franza, B.R. and Greene, W.C. (1988). The same inducible nuclear protein regulates mitogen activation of both the interleukin-2 receptor-α gene and type I HIV. *Cell* **53**:827-836.

Bornstein, P., McKay, J., Morishima, J.K., Devarayalu, S. and Gelinas, R.E. (1987). Regulatory elements in the first intron contribute to transcriptional control of the human α1(I) collagen gene. *Proc. Natl. Acad. Sci. USA* **84**:8869-8873.

Bowlus, C.L., McQuillan, J.J. and Dean, D.C. (1991). Characterization of three different elements in the 5'-flanking region of the fibronectin gene which mediate a transcriptional response to cAMP. *J. Biol. Chem.* **266**:1122-1127.

Boyle, W.J., Smeal, T., Defize, L.H.K., Angel, P., Woodgett, J.R., Karin, M. and Hunter, T. (1991). Activation of protein kinase C decreases phosphorylation of c-*jun* at sites that negatively regulate its DNA-binding activity. *Cell* **64**:573-584.

Brand, A.H., Breeden, L., Abraham, J., Sternglanz, R. and Nasmyth, K. (1985). Characterization of a "silencer" in yeast: a DNA sequence with properties opposite to those of a transcriptional enhancer. *Cell* **41**:41-48.

Bresnick, E.H., John, S., Berard, D.S., LeFebvre, P. and Hager, G.L. (1990). Glucocorticoid receptor-dependent disruption of a specific nucleosome on the mouse mammary tumor virus promoter is prevented by sodium butyrate. *Proc. Natl. Acad. Sci. USA* **87**:3977-3981.

Brunet, J.F., Dosseto, M., Denizot, F., Matter, M.-G., Clark, W.R., Haqqi, T.M., Ferrier, P., Nabholz, M., Schmitt-Verhulst, A.-M., Luciani, M.-F. and Golstein, P. (1986). The inducible cytotoxic T-lymphocyte-associated gene transcript CTLA-1 sequence and gene localization to mouse chromosome 14. *Nature* **322**:268-271.

Candido, E.P.M., Reeves, R. and Davie, J.R. (1978). Sodium butyrate inhibits histone deacetylation in cultured cells. *Cell* **14**:105-113.

Cantrell, D., Davies, A.A., Londei, M., Feldman, M. and Crumpton, M.J. (1987). Association of phosphorylation of the T3 antigen with immune activation of T lymphocytes. *Nature* **325**:540-542.

Carpén, O. and Saksela, E. (1988). Directed exocytosis in the NK cell-mediated cytotoxicity. A review. *Nat. Immun. Cell Growth Regul.* **7**:1-12.

Charollais, R.-H., Buquet, C. and Mester, J. (1990). Butyrate blocks the accumulation of CDC2 mRNA in late G1 phase but inhibits both the early and late G1 progression in chemically transformed mouse fibroblasts BP-A31. *J. Cell. Physiol.* **145**:46-52.

Chodosh, L.A., Baldwin, A.S., Carthew, R.W. and Sharp, P.A. (1988). Human CCAAT-binding proteins have heterologous subunits. *Cell* **53**:11-24.

Chou, J.Y., Sartwell, A.D., Lei, K.-J. and Plouzek, C.A. (1990). Effects of sodium butyrate on the synthesis of human pregnancy-specific β1-glycoprotein. *J. Biol. Chem.* **265**:8788-8794.

Churchill, M.E.A. and Travers, A.A. (1991). Protein motifs that recognize structural features of DNA. *Trends Biochem. Sci.* **16**:92-97.

Clevers, H., Lonberg, N., Dunlap, S., Lacy, E. and Terhorst, C. (1989). An enhancer located in a CpG-island 3' to the TCR/CD3-γ gene confers T lymphocyte-specificity to its promoter. *EMBO J.* **8**:2527-2535.

Cockerill, P.N. and Garrard, W.T. (1986). Chromosomal loop anchorage of the kappa immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites. *Cell* **44**:273-283.

Cockerill, P.N., Yuen, M.-H. and Garrard, W.T. (1987). The enhancer of the immunoglobulin heavy chain locus is flanked by presumptive chromosomal loop anchorage elements. *J. Biol. Chem.* **262**:5394-5397.

Cohen, R.B., Boal, T.R. and Safer, B. (1990). Increased eIF-2α expression in mitogen-activated primary T lymphocytes. *EMBO J.* **9**:3831-3837.

Colvin, R.B. (1990). Cellular and molecular mechanisms of allograft rejection. *Ann. Rev. Med.* **41**:361-375.

Comb, M., Mermod, N., Hyman, S.E., Pearlberg, J., Ross, M.E. and Goodman, H.M. (1988). Proteins bound at adjacent DNA elements act synergistically to regulate human proenkephalin cAMP inducible transcription. *EMBO J.* **7**:3793-3805.

Costa, R.H., Grayson, D.R., Xanthopoulos, K.G., Darnell Jr., J.E. (1988). A liver-specific DNA binding protein recognizes multiple nucleotide sites in regulatory regions of transthyretin, α1-antitrypsin, albumin, and simian virus 40 genes. *Proc. Natl. Acad. Sci. USA* **85**:3840-3844.

Cotner, T., Williams, J.M., Christenson, L., Shapiro, H.M., Strom, T.B. and Strominger, J. (1983). Simultaneous flow cytometric analysis of human T cell activation antigen expression and DNA content. *J. Exp. Med.* **157**:461-472.

Crabtree, G.R. (1989). Contingent genetic regulatory events in T lymphocyte activation. *Science* **243**:355-361.

Craigen, W.J. and Caskey, C.T. (1987). Translational frame shifting: where will it stop. *Cell* **50**:1-2.

Crosby, J.L., Bleackley, R.C. and Nadeau, J.H. (1990). A complex of serine protease genes expressed preferentially in cytotoxic T lymphocytes is closely linked to the T-cell receptor α- and δ-chain genes on mouse chromosome 14. *Genomics* **6**:252-259.

Davis, M.M. (1988). T cell antigen receptor genes. In Molecular Immunology. (B.D. Hames and D.M. Glover, eds). IRL Press, Washington, pp. 61-79.

Davis, M.M. (1990). T cell receptor gene diversity and selection. *Ann. Rev. Biochem.* **59**:475-496.

Denner, L. A., Weigel, N.L., Maxwell, B.L., Schrader, W.T. and O'Malley, B.W. (1990). Regulation of progesterone receptor-mediated transcription by phosphorylation. *Science* **250**:1740-1743.

Dennert, G. and Podack, E.R. (1983). Cytolysis by H2 specific T-killer cells: assembly of tubular complexes during the lytic reaction. *J. Exp. Med.* **157**:1483-1495.

Desplan, C., Theis, J. and O'Farrell, P.H. (1988). The sequence specificity of homeodomain-DNA interaction. *Cell* **54**:1081-1090.

Deutsch, P.J., Hoeffler, J.P., Jameson, J.L. and Habener, J.F. (1988). Cyclic AMP and phorbol ester-stimulated transcription mediated by similar DNA elements that bind distinct proteins. *Proc. Natl. Acad. Sci. USA* **85**:7922-7926.

Diamond, D.J., Nelson, F.B. and Reinherz, E.L. (1989). Lineage-specific expression of a T cell receptor variable gene promoter controlled by upstream sequences. *J. Exp. Med.* **169**:1213-1231.

Diamond, M.I., Miner, J.N., Yoshinaga, S.K. and Yamamoto, K.R. (1990). Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. *Science* **249**:1266-1272.

Dorn, A., Bollekens, J., Stauls, A., Benoist, C. and Mathis, D. (1987). A multiplicity of CCAAT box-binding proteins. *Cell* **50**:863-872.

Dorner, A.J., Wasley, L.C. and Kaufman, R.J. (1989). Increased synthesis of secreted proteins induces expression of glucose-regulated proteins in butyrate-treated Chinese hamster ovary cells. *J. Biol. Chem.* **264**:20602-20607.

Dourmashkin, R.R., Deteix, P., Simone, C.B. and Henkart, P. (1980). Electron microscopic demonstration of lesions on target cell membranes associated with antibody-dependent cellular cytotoxicity. *Clin. Exp. Immunol.* **43**:554-560.

Doyle, C. and Strominger, J.L. (1987). Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature* **330**:256-259.

Duke, R.C., Chervenak, R. and Cohen, J.J. (1983). Endogenous endonuclease-induced DNA fragmentation: an early event in cell-mediated cytolysis. *Proc. Natl. Acad. Sci. USA* **80**:6361-6365.

Duke, R.C., Sellins, K.S. and Cohen, J.J. (1988). Cytotoxic lymphocyte-derived lytic granules do not induce DNA fragmentation in target cells. *J. Immunol.* **141**:2191-2194.

Duvall, E. and Wyllie, A.H. (1986). Death and the cell. *Immunol. Today* **7**:115-119.

Eardley, D.D. and Koshland, M.E. (1991). Glycosylphosphatidylinositol: a candidate system for interleukin-2 signal transduction. *Science* **251**:78-81.

Efrat, S. and Kaempfer, R. (1984). Control of biologically active interleukin 2 messenger RNA formation in induced human lymphocytes. *Proc. Natl. Acad. Sci. USA* **81**:2601-2605.

Elgin, S.C.R. (1988). The formation and function of DNAse I hypersensitivity sites in the process of gene activation. *J. Biol. Chem.* **263**:19259-19262.

Fascher, K.-D., Schmitz, J. and Hörz, W. (1990). Role of trans-activating proteins in the generation of active chromatin at the PHO5 promoter in *S. cerevisiae. EMBO J.* **9**:2523-2528.

Feng, P., Ohlsson, M. and Ny, T. (1990). The structure of the TATA-less rat tissue-type plasminogen activator gene. *J. Biol. Chem.* **265**:2022-2027.

Ferguson, T.A., Ptak, W., Iverson, G.M. and Flood, P. (1988). The role of suppression in immunoregulation: *in vivo* analysis using a monoclonal antibody to T suppressor factors. *Eur. J. Immunol.* **18**:1179-1185.

Ferluga, J., Asherson, G.L. and Becker, E.L. (1982). The effect of organophosphorous inhibitors, para-nitrophenol and cytochalasin B on cytotoxic killing of tumor cells by immune spleen cells, and the effect of shaking. *Immunology* **23**:577-590.

Filippini, A., Taffs, R.E., Agui, T. and Sitkovsky, M.V. (1990a). Ecto-ATPase activity in cytolytic T-lymphocytes. Protection from the cytolytic effects of extracellular ATP. *J. Biol. Chem.* **265**:334-340.

Filippini, A., Taffs, R.E. and Sitkovsky, M.V. (1990b). Extracellular ATP in T-lymphocyte activation: possible role in effector functions. *Proc. Natl. Acad. Sci. USA* **87**:8267-8271.

Ford, C.E., Micklem, H.S., Evans, E.P., Gray, J.G. and Ogden, D.A. (1966). The inflow of bone marrow cells to the thymus: studies with part-body irradiated mice injected with chromosome-marked bone marrow and subjected to antigenic stimulation. *Ann. N.Y. Acad. Sci.* **129**:283-296.

Fraser, J.D., Goldsmith, M.A. and Weiss, A. (1989). Ligand-induced association between the T-cell antigen receptor and two glycoproteins. *Proc. Natl. Acad. Sci. USA* **86**:7133-7137.

Fraser, J.D., Irving, B.A., Crabtree, G.R. and Weiss, A. (1991). Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. *Science* **251**:313-316.

Fruth, U., Sinigaglia, F., Schlesier, M., Kilgus, J., Kramer, M.D. and Simon, M.M. (1987). A novel serine proteinase (HuTSP) isolated from a cloned human CD8$^+$ cytolytic T cell line is expressed and secreted by activated CD4$^+$ and CD8$^+$ lymphocytes. *Eur. J. Immunol.* **17**:1625-1633.

Fujita, T., Shibuya, H., Ohashi, T., Yamanishi, K. and Taniguchi, T. (1986). Regulation of human interleukin-2 gene: functional DNA sequences in the 5' flanking region for the gene expression in activated T lymphocytes. *Cell* **46**:401-407.

Gabert, J., Langlet, C., Zamoyska, R., Parnes, J.R., Schmitt-Verhulst, A.-M. and Malissen, B. (1987). Reconstitution of MHC class I specificity by transfer of the T cell receptor and Lyt-2 genes. *Cell* **50**:545-554.

Gasser, S.M. and Laemmli, U.K. (1986). Cohabitation of scaffold binding regions with upstream/enhancer elements of three developmentally regulated genes of *D. melanogaster*. *Cell* **46**:521-530.

Geiger, B., Rosen, D. and Berke, G. (1982). Spatial relationships of microtubule-organizing centers and the contact area of cytotoxic T lymphocytes and target cells. *J. Cell. Biol.* **95**:137-143.

Georgopoulos, K., Galson, D. and Terhorst, C. (1990). Tissue-specific nuclear factors mediate expression of the CD3 δ gene during T cell development. *EMBO J.* **9**:109-115.

Georgopoulos, K., van den Elsen, P., Bier, E., Maxam, A. and Terhorst, C. (1988). A T cell-specific enhancer is located in a DNAse 1-hypersensitive area at the 3' end of the CD3 δ gene. *EMBO J.* **7**:2401-2407.

Gershenfeld, H.K., Hershberger, R.J., Shows, T.B. and Weissman, I.L. (1988). Cloning and chromosomal assignment of a human cDNA encoding a T cell and natural killer cell-specific trypsin-like serine protease. *Proc. Natl. Acad. Sci. USA* **85**:1184-1188.

Gershenfeld, H.K. and Weissman, I.L. (1986). Cloning of a cDNA for a T cell-specific serine protease from a cytotoxic T lymphocyte. *Science* **232**:854-858.

Ghosh, H. and Baltimore, D. (1990). Activation *in vitro* of NFκB by phosphorylation of its inhibitor IκB. *Nature* **344**:678-682.

Glaichenhaus, N., Shastri, N., Littman, D.R. and Turner, J.M. (1991). Requirement for association of p56^{lck} with CD4 in antigen-specific signal transduction in T cells. *Cell* **64**:511-520.

Gleich, G.J. and Adolphson, C.R. (1986). The eosinophil leukocyte: structure and function. *Adv. Immunol.* **39**:177-253

Glineur, C., Zenke, M., Beug, H. and Ghysdael, J. (1990). Phosphorylation of the v-*erbA* protein is required for its function as an oncogene. *Genes Dev.* **4**:1663-1676.

Gonzalez, G.A., Menzel, P., Leonard, J., Fischer, W.H. and Montminy, M.R. (1991). Characterization of motifs which are critical for activity of the cyclic AMP-responsive transcription factor CREB. *Mol. Cell. Biol.* **11**:1306-1312.

Gonzalez, G.A. and Montminy, M.R. (1989). Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59**:675-680.

Goodbourn, S. (1990). Negative regulation of transcriptional initiation in eukaryotes. *Biochem. Biophys. Acta* **1032**:53-77.

Goodbourn, S. and Maniatis, T. (1988). Overlapping positive and negative regulatory domains of the human β-interferon gene. *Proc. Natl. Acad. Sci. USA* **85**:1447-1451.

Gordon, E.L., Pearson, J.D. and Slakey, L.L. (1986). The hydrolysis of extracellular adenine nucleotides by cultured endothelial cells from pig aorta. *J. Biol. Chem.* **261**:15496-15504.

Gordon, J.R., Burd, P.R. and Galli, S.J. (1990). Mast cells as a source of multifunctional cytokines. *Immunol. Today* **11**:458-464.

Gray, L.S., Gnarra, J.R. and Engelhard, V.H. (1987). Demonstration of a calcium influx in cytolytic T lymphocytes in response to target cell binding. *J. Immunol.* **138**:63-69.

Greaves, D.R., Wilson, F.D., Lang, G. and Kioussis, D. (1989). Human CD2 3'-flanking sequences confer high-level, T cell-specific, position-independent gene expression in transgenic mice. *Cell* **56**:979-986.

Greenberg, M.E., Hermanowski, A.L. and Ziff, E.B. (1986). Effect of protein synthesis inhibitors on growth factor activation of c-fos, c-myc, and actin gene transcription. *Mol. Cell. Biol.* **6**:1050-1057.

Gromkowski, S.H., Brown, T.C., Masson, D. and Tschopp, J. (1988). Lack of DNA degradation in target cells lysed by granules derived from cytolytic T lymphocytes. *J. Immunol.* **141**:774-778.

Gross, D.S. and Garrard, W.T. (1988). Nuclease hypersensitive sites in chromatin. *Ann. Rev. Biochem.* **57**:159-197.

Haddad, P., Jenne, D., Tschopp, J., Clement, M.V., Mathieumahul, D. and Sasportes, M. (1991). Structure and evolutionary origin of the human granzyme-H gene. Intern. Immunol. **3**:57-66.

Hall, D.J., Alberta, J.A. and Stiles, C.D. (1989). Labile repressors are involved in the transcriptional control of PDGF-responsive genes.*Oncogene Res.* **4**:177-184.

Hameed, A., Lowrey, D.M., Lichtenheld, M. and Podack, E.R. (1988). Characterization of three serine esterases isolated from human IL2 activated killer cells. *J. Immunol.* **141**:3142-3147.

Hammer, R.E., Krumlauf, R., Camper, S.A., Brinster, R.L., Tilghman, S.M. (1987). Diversity of α-fetoprotein gene expression in mice is generated by a combination of separate enhancer elements. *Science* **235**:53-58.

Hanson, R.D., Connolly, N.L., Burnett, D., Campbell, E.J., Senior, R.M. and Ley, T.J. (1990). Developmental regulation of the human cathepsin G gene in myelomonocytic cells. *J. Biol. Chem.* **265**:1524-1530.

Hanson, R.D., Hohn, P.A., Popescu, N.C. and Ley, T.J. (1990). A cluster of hematopoietic serine protease genes is found on the same chromosomal band as the human α/δ T-cell receptor locus. *Proc. Natl. Acad. Sci. USA* **87**:960-963.

Harrison, S.C. and Aggarwal, A.K. (1990). DNA recognition by proteins with the helix-turn-helix motif. *Ann. Rev. Biochem.* **59**:933-969.

Hatakeyama, M., Tsudo, M., Minamoto, S., Kono, T., Doi, T., Miyata, T., Miyasaka, M. and Taniguchi, T. (1989). Interleukin-2 receptor β chain gene: generation of three receptor forms by cloned human a and b chain cDNA's. *Science* **244**:551-556.

Hayes, M.P., Berrebi, G.A. and Henkart, P.A. (1989). Induction of target cell DNA release by the cytotoxic T lymphocyte granule protease granzyme *A. J. Exp. Med.* **170**:933-946.

Haynes, B.F., Denning, S.M., Singer, K.H. and Kurtzberg, J. (1989). Ontogeny of T-cell precursors: a model for the initial stages of human T-cell development. *Immunol. Today* **10**:87-91.

Heikkila, R., Schwab, G., Wickstrom, E., Loke, S.L., Pluznik, D.H., Watt, R. and Neckers, L.M. (1987). A c-myc antisense oligodeoxynucleotide inhibits entry into S phase but not progress from G0 to G1. *Nature* **328**:445-449.

Held, W., MacDonald, H.R., Weissman, I.L., Hess, M.W. and Müeller, C. (1990). Genes encoding tumor necrosis factor α and granzyme A are expressed during development of autoimmune diabetes. *Proc. Natl. Acad. Sci. USA* **87**:2239-2243.

Henkart, M.P. and Henkart P.A. (1982). Lymphocyte mediated cytolysis as a secretory phenomenon. In *Mechanisms of cell mediated cytotoxicity.* (W.R. Clark and P. Goldstein, eds), Plenum Publishing Corp. pp. 227-242.

Henkart, P.A. (1985). Mechanisms of lymphocyte-mediated cytotoxicity. *Ann. Rev. Immunol.* **3**:31-58.

Henkart, P.A., Millard, P.J., Reynolds, C.J. and Henkart, M.P. (1984). Cytolytic activity of purified cytoplasmic granules from cytotoxic rat large granular lymphocyte tumors. *J. Exp. Med.* **160**:75-93.

Henney, C.S. (1973). Studies on the mechanism of T cell-mediated cytolysis. *Transplant Rev.* **17**:37-70.

Henney, C.S. (1975). T cell mediated cytolysis: consideration of the role of a soluble mediator. *J. Reticuloendothel. Soc.* **17**:231-235.

Hershey, J.W.B. (1989). Protein phosphorylation controls translation rates. *J. Biol. Chem.* **264**:20823-20826.

Hiserodt, J.C., Britvan, L.J. and Targan, S.R. (1982). Differential effects of various pharmacological agents on the cytolytic reaction mechanisms of the human natural killer lymphocyte: further resolution of programming for lysis and KCIL into discrete stages. *J. Immunol.* **129**:2266-2270.

Ho, I.-C., Bhat, N.K., Gottschalk, L.R., Lindsten, T., Thompson, C.B., Papas, T.S. and Leiden, J.M. (1990). Sequence-specific binding of human Ets-1 to the T cell receptor α gene enhancer. *Science* **250**:814-818.

Ho, I.-C., Yang, L.-H., Morle, G. and Leiden, J.M. (1989). A T-cell-specific transcriptional enhancer element 3' of Cα in the human T-cell receptor α locus. *Proc. Natl. Acad. Sci. USA* **86**:6714-6718.

Hofmann, J.F.-X., Laroche, T., Brand, A.H. and Gasser, S.M. (1989). RAP-1 factor is necessary for DNA loop formation *in vitro* at the silent mating type locus HML. *Cell* **57**:725-737.

Horton, W., Miyashita, T., Kohno, L., Hassell, J.R. and Yamada, Y. (1987). Identification of a phenotype-specific enhancer in the first intron of the rat collagen II gene. *Proc. Natl. Acad. Sci. USA* **84**:8864-8868.

Horvat, B., Ferguson, T.A., Rodriguez, A., Reuter, P., Friedman, A. and Flood, P.M. (1989). The role of suppressor T cells in immunologic responses analyzed with monoclonal anti-T suppressor cell antibodies. In *Progress in Leukocyte Biology vol. 9. Cellular Basis of Immune Modulation.* (J.G. Kaplan, D.R. Green and R.C. Bleackley, eds), New York, Alan R. Liss, pp. 353-366.

Howell, D.M. and Martz, E. (1988). Low calcium concentrations support killing by some but not all cytolytic T lymphocytes, and reveal inhibition of a postconjugation step by calcium antagonists. *J. Immunol.* **140**:1982-1988.

Hudig, D., Haverty, T., Fulcher, C., Redelman, D. and Mendelsohn, J. (1981). Inhibition of human natural cytotoxicity by macromolecular antiproteases. *J. Immunol.* **126**:1569-1574.

Hudig, D., Redelman, D. and Minning, L.L. (1984). The requirement for proteinase activity for human lymphocyte-mediated natural cytotoxicity (NK): evidence that the proteinase is serine dependent and has aromatic amino acid specificity of cleavage. *J. Immunol.* **133**:2647-2654.

Imagawa, M., Osada, S., Okuda, A. and Muramatsu, M. (1991). Silencer binding proteins function on multiple cis-elements in the glutathione transferase P gene. *Nucleic Acids Res.* **19**:5-10.

Isakov. N., Mally, M.I., Scholz, W. and Altman, A. (1987). T-lymphocyte activation: the role of protein kinase C and the bifurcating inositol phospholipid signal transduction pathway. *Immunol. Rev.* **95**:89-111.

Ishida, I., Verbeek, S., Bonneville, M., Itohara, S., Berns, A. and Tonegawa, S. (1990). T-cell receptor γδ and γ transgenic mice suggest a role of a γ gene silencer in the generation of αβ T cells. *Proc. Natl. Acad. Sci. USA* **87**:3067-3071.

Ishii, S., Xu, Y.-H., Stratton, R.T., Roe, B.A., Merlino, G.T. and Pastan, I. (1985). Characterization and sequence of the promoter region of the human epidermal growth factor receptor gene. *Proc. Natl. Acad. Sci. USA* **82**:4920-4924.

Jackson, R.J. and Standart, N. (1990). Do the poly(A) tail and 3' untranslated region control mRNA translation? *Cell* **62**:15-24.

Jackson, S.P., MacDonald, J.J., Lees-Miller, S. and Tjian, R. (1990). GC box binding induces phosphorylation of Sp1 by a DNA-dependent protein kinase. *Cell* **63**:155-165.

Jakobovits, E.B., Schlokat, U., Vannice, J.L., Derynck, R. and Levinson, A.D. (1988). The human transforming growth factor-α promoter directs transcription initiation from a single site in the absence of a TATA sequence. *Mol. Cell. Biol.* **8**:5549-5554.

Jenne, D.E., Masson, D., Zimmer, M., Haefliger, J.-A., Li, W.-H. and Tschopp, J. (1989). Isolation and complete structure of the lymphocyte serine protease granzyme G, a novel member of the granzyme multigene family in murine cytolytic T lymphocytes. Evolutionary origin of lymphocyte proteases. *Biochemistry* **28**:7953-7961.

Jenne, D., Rey, C., Haefliger, J.-A., Qiao, B.-Y., Groscurth, P. and Tschopp, J. (1988). Identification and sequencing of cDNA clones encoding the granule-associated serine proteases granzymes D, E, and F of cytolytic T lymphocytes. *Proc. Natl. Acad. Sci. USA* **85**:4814-4818.

Jenne, D.E. and Tschopp, J. (1988). Granzymes, a family of serine proteases released from granules of cytolytic T lymphocytes upon T cell receptor stimulation. *Immunol. Rev.* **103**:53-71.

Jiang, S., Ojcius, D.M., Persechini, P.M. and Young, J.D.-E (1990). Resistance of cytolytic lymphocytes to perforin-mediated killing. Inhibition of perforin binding activity by surface membrane proteins. *J. Immunol.* **144**:998-1003.

Joag, S., Zychlinsky, A. and Young, J.D.-E (1989). Mechanisms of lymphocyte-mediated lysis. *J. Cell. Biochem.* **39**:239-252.

Johnson, P.F. and McKnight, S.L. (1989). Eukaryotic transcriptional regulatory proteins. *Ann. Rev. Biochem.* **58**:799-839.

Jones, N.C., Rigby, P.W. and Ziff, E.B. (1988). Trans-acting protein factors and the regulation of eukaryotic transcription: lessons from studies on DNA tumor viruses. *Genes and Dev.* **2**:267-281.

Kadesch, T., Zervos, P. and Ruezinsky, D. (1986). Functional analysis of the murine IgH enhancer: evidence for negative control of cell-type specificity. *Nucleic Acids Res.* **14**:8209-8221.

Kamakaka, R.T. and Thomas, J.O. (1990). Chromatin structure of transcriptionally competent and repressed genes. *EMBO J.* **9**:3997-4006.

Kamata, N., Jotte, R.M. and Holt, J.T. (1991). Myristylation alters DNA-binding activity and transactivation of FBR (gag-fos) protein. *Mol. Cell. Biol.* **11**:765-772.

Kao, R.C., Wehner, N.G., Skubitz, K.M., Gray, B.H. and Hoidal, J.R. (1988). Proteinase 3. A distinct human polymorphonuclear leukocyte proteinase that produces emphysema in hamsters. *J. Clin. Invest.* **82**:1963-1973.

Kargi, H.A., Campbell, E.J. and Kuhn III, C. (1990). Elastase and cathepsin G of human monocytes: heterogeneity and subcellular localization to peroxidase positive granules. *J. Histochem. Cytochem.* **38**:1179-1186.

Karim, F.D., Urness, L.D., Thummel, C.S., Klemsz, M.J., McKercher, S.R., Celada, A., van Beveren, C., Maki, R.A., Gunther, C.V., Nye, J.A. and Graves, B.J. (1990). The Ets-domain: a new DNA-binding motif that recognizes a purine-rich core DNA sequence. *Genes Dev.* **4**:1451-1453.

Karsenty, G. and de Crombrugghe, B. (1990). Two different negative and one positive regulatory factors interact with a short promoter segment of the $\alpha 1$(I) collagen gene. *J. Biol. Chem.* **265**:9934-9942.

Klein, J.L., Shows, T.B., Dupont, B. and Trapani, J.A. (1989). Genomic organization and chromosomal assignment for a serine protease gene (CSP-B) expressed by human cytotoxic lymphocytes. *Genomics* **5**:110-117.

Koizumi, H., Liu, C.-C., Zheng, L.M., Joag, S.V., Bayne, N.K., Holoshitz, J. and Young, J. D.-E (1991). Expression of perforin and serine esterases by human γ/δ T cells. *J. Exp. Med.* **173**:499-502.

Kouzarides, T. and Ziff, E. (1988). The role of the leucine zipper in the fos-jun interaction. *Nature* **336**:646-651.

Krähenbühl, O. and Tschopp, J. (1990). Involvement of granule proteins in T cell-mediated cytolysis. *Nat. Immun. Cell Growth Regul.* **9**:274-282.

Kramer, M.D. and Simon, M.M. (1987). Are proteinases functional molecules of T lymphocytes? *Immunol. Today* **8**:140-142.

Kranz, D.M. and Eisen, H.N. (1987). Resistance of cytotoxic T lymphocytes to lysis by a clone of cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* **84**:3375-3379.

Kruh, J. (1982). Effects of sodium butyrate, a new pharmacological agent, on cells in culture. *Mol. Cell. Biochem.* **42**:65-82.

Kumar, V., Kono, D.H., Urban, J.L. and Hood, L. (1989). The T cell receptor repertoire and autoimmune diseases. *Ann. Rev. Immunol.* **7**:657-682.

Kupfer, A. and Dennert, G. (1984). Reorientation of the microtubule-organizing center and the Golgi apparatus in cloned cytotoxic lymphocytes triggered by binding to lysable target cells. *J. Immunol.* **133**:2762-2766.

Kupfer, A., Dennert, G. and Singer, S.J. (1985). The reorientation of the Golgi apparatus and the microtubule-organizing center in the cytotoxic effector is a prerequisite in the lysis of bound target cells. *J. Mol. Cell. Immunol.* **2**:37-49.

Kwon, B.S., Kim, G.S., Prystowsky, M.B., Lancki, D.W., Sabath, D.E., Pan, J. and Weissman, S.M. (1987). Isolation and initial characterization of multiple species of T-lymphocyte subset cDNA clones. *Proc. Natl. Acad. Sci. USA* **84**:2896-2900.

Lake, R.A., Wotton, D. and Owen, M.J. (1990). A 3' transcriptional enhancer regulates tissue-specific expression of the human CD2 gene. *EMBO J.* **9**:3129-3136.

Lamph, W.W., Dwarki, V.J., Ofir, R., Montminy, M. and Verma, I.M. (1990). Negative and positive regulation by transcription factor cAMP response element-binding protein is modulated by phosphorylation. *Proc. Natl. Acad. Sci. USA* **87**:4320-4324.

Landschulz, W.H., Johnson, P.F., McKnight, S.L. (1988). The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* **240**:1759-1764.

Lazar, M.A. (1990). Sodium butyrate selectively alters thyroid hormone receptor gene expression in GH3 cells. *J. Biol. Chem.* **265**:17474-17477.

Lenardo, M.J. and Baltimore, D. (1989). NF-κB: a pleiotropic mediator of inducible and tissue-specific gene control. *Cell* **58**:227-229.

Lenardo, M.J., Staudt, L., Robbins, P., Kuang, A., Mulligan, R.C. and Baltimore, D. (1989). Repression of the IgH enhancer in teratocarcinoma cells associated with a novel octamer factor. *Science* **243**:544-546.

Lennon, G.G. and Perry, R.P. (1985). Cμ-containing transcripts initiate heterogeneously within the IgH enhancer region and contain a novel 5'-nontranslatable exon. *Nature* **318**:475-478.

Levy-Wilson, B. (1981). Enhanced phosphorylation of high-mobility-group proteins in nuclease-sensitive mononucleosomes from butyrate-treated HeLa cells. *Proc. Natl. Acad. Sci. USA* **78**:2189-2193.

Lewin, B. (1990). Commitment and activation at Pol II promoters: a tail of protein-protein interactions. *Cell* **61**:1161-1164.

Lichtenheld, M.G., Olsen, K.J., Lu, P., Lowrey, D.M., Hameed, A., Hengartner, H., Podack, E.R. (1988). Structure and function of human perforin. *Nature* **335**:448-451.

Lichtenheld, M.G. and Podack, E.R. (1989). Structure of the human perforin gene. A simple gene organization with interesting potential regulatory sequences. *J. Immunol.* **143**:4267-4274.

Liu, C.-C., Perussia, B., Cohn, Z.A. and Young, J.D.-E (1986). Identification and characterization of a pore-forming protein of human peripheral blood natural killer cells. *J. Exp. Med.* **164**:2061-2076.

Liu, C.-C., Steffen, M., King, F. and Young, J.D.-E (1987). Identification, isolation, and characterization of a novel cytotoxin in murine cytolytic lymphocytes. *Cell* **51**:393-403.

Liu, Q., Tini, M., Tsui, L.-C. and Breitman, M.L. (1991). Interaction of a lens cell transcription factor with the proximal domain of the mouse γF-crystallin promoter. *Mol. Cell. Biol.* **11**:1531-1537.

Lobe, C.G., Finlay, B.B., Paranchych, W., Paetkau, V. and Bleackley, R.C. (1986a). Novel serine proteases encoded by two cytotoxic T lymphocyte-specific genes. *Science* **232**:858-861.

Lobe, C.G., Havele, C., and Bleackley, R.C. (1986b). Cloning of two genes that are specifically expressed in activated cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* **83**:1448-1452.

Lobe, C.G., Upton, C., Duggan, B., Ehrman, N., Letellier, M., Bell, J., McFadden, G. and Bleackley, R.C. (1988). Organization of two genes encoding cytotoxic T lymphocyte-specific serine proteases CCPI and CCPII. *Biochemistry* **27**:6941-6946.

Lotzová, E. (1990). Role of human circulating and tumor-infiltrating lymphocytes in cancer defense and treatment. *Nat. Immun. Cell Growth Regul.* **9**:253-264.

Lotzová, E. and Herberman, R.B. (1987). Reassessment of LAK phenomenology: a review. *Nat. Immun. Cell Growth Regul.* **6**:109-115.

Lowenthal, J.W., Ballard, D.W., Böhnlein, E. and Greene, W.C. (1989). Tumor necrosis factor induces proteins that bind specifically to κB-like enhancer elements and regulate interleukin 2 receptor α-chain gene expression in primary human T lymphocytes. *Proc. Natl. Acad. Sci. USA* **86**:2331-2335.

Lowrey, D.M., Aebischer, T., Olsen, K., Lichtenheld, M., Rupp, F. and Podack, E.R. (1989). Cloning, analysis, and expression of murine perforin 1 cDNA, a component of cytolytic T-cell granules with homology to complement component C9. *Proc. Natl. Acad. Sci. USA* **86**:247-251.

Maity, S.N., Golumbek, P.T., Karsenty, G. and de Crombrugghe, B. (1988). Selective activation of transcription by a novel CCAAT binding factor. *Science* **241**:582-585.

Mandal, N., Su, W., Haber, R., Adhya, S. and Echols, H. (1990). DNA looping in cellular repression of transcription of the galactose operon. *Genes Dev.* **4**:410-418.

Maniatis, T., Goodbourn, S. and Fischer, J.A. (1987). Regulation of inducible and tissue-specific gene expression. *Science* **236**:1237-1245.

Maniatis, T. (1991). Mechanisms of alternative pre-mRNA splicing. *Science* **251**:33-34.

Masson, D. and Tschopp, J. (1987). A family of serine esterases in lytic granules of cytolytic T-lymphocytes. *Cell* **49**:679-685.

Masson, D., Zamai, M. and Tschopp, J. (1986). Identification of granzyme A isolated from cytotoxic T lymphocyte granules as one of the proteases encoded by CTL-specific genes. *FEBS Lett.* **208**:84-88.

Matter, A. (1975). A study of proteolysis as a possible mechanism for T cell-mediated target cell lysis. *Scand. J. Immunol.* **4**:349-356.

McDougall, S., Peterson, C.L. and Calame, K. (1988). A transcriptional enhancer 3' of Cβ2 in the T cell receptor β locus. *Science* **241**:205-208.

McKnight, S. and Tjian, R. (1986). Transcriptional selectivity of viral genes in mammalian cells. *Cell* **46**:795-805.

Means, A.L. and Farnham, P.J. (1990). Transcription initiation from the dihydrofolate reductase promoter is positioned by HIP1 binding at the initiation site. *Mol. Cell. Biol.* **10**:653-661.

Meier, M., Kwong, P.C., Frégeau, C.J., Atkinson, E.A., Burrington, M., Ehrman, N., Sorenson, O., Lin, C.C., Wilkins, J. and Bleackley, R.C. (1990). Cloning of a gene that encodes a new member of the human cytotoxic cell protease family. *Biochemistry* **29**:4042-4049.

Merino, A., Buckbinder, L., Mermelstein, F.H. and Reinberg, D. (1989). Phosphorylation of cellular proteins regulates their binding to the cAMP response element. *J. Biol. Chem.* **264**:21266-21276.

Meyer, R., Hatada, E.N., Hohmann, H.-P., Haiker, M., Bartsch, C., Röthlisberger, U., Lahm, H.-W., Schlaeger, E.J., van Loon, A.P.G.M. and Scheidereit, C. (1991). Cloning of the DNA-binding subunit of human nuclear factor κB: the level of its mRNA is strongly regulated by phorbol ester or tumor necrosis factor α. *Proc. Natl. Acad. Sci. USA* **88**:966-970.

Mickley, L.A., Bates, S.E., Richert, N.D., Currier, S., Tanaka, S., Foss, F., Rosen, N. and Fojo, A.T. (1989). Modulation of the expression of a multidrug resistance gene (mdr-1/P-glycoprotein) by differentiating agents. *J. Biol. Chem.* **264**:18031-18040.

Mirkovitch, J., Mirault, M.-E., Laemmli, U.K. (1984). Organization of the higher-order chromatin loop: specific DNA attachment sites on nuclear scaffold. *Cell* **39**:223-232.

Morse, R.H. and Simpson, R.T. (1988). DNA in the nucleosome. *Cell* **54**:285-287.

Mosmann, T.R. and Coffman, R.L. (1989). $T_{H}1$ and $T_{H}2$ cells: different patterns of lymphokine secretion lead to different functional properties. *Ann. Rev. Immunol.* **7**:145-173.

Müeller, C., Gershenfeld, H.K., Lobe, C.G., Okada, C.Y., Bleackley, R.C. and Weissman, I.L. (1988a). A high proportion of T lymphocytes that infiltrate H-2-incompatible heart allografts *in vivo* express genes encoding cytotoxic cell-specific serine proteases, but do not express the MEL 14-defined lymph node homing receptor. *J. Exp. Med.* **167**:1124-1136.

Müeller, C., Gershenfeld, H.K. and Weissman, I.L. (1988b). Activation of CTL-specific genes during cell-mediated cytolysis *in vivo*: expression of the HF gene analyzed by *in situ* hybridization. *Immunol. Rev.* **103**:73-85.

Müeller, C., Kägi, D., Aebischer, T., Odermatt, B., Held, W., Podack, E.R., Zinkernagel, R.M. and Hengartner, H. (1989). Detection of perforin and granzyme A mRNA in infiltrating cells during infection of mice with lymphocytic choriomeningitis virus. *Eur. J. Immunol.* **19**:1253-1259.

Mukherjee, S., Erickson, H. and Bastia, D. (1988). Enhancer-origin interaction in plasmid R6K involves a DNA loop mediated by initiator protein. *Cell* **52**:375-383.

Müller-Eberhard, H.J. (1988). The molecular basis of target cell killing by human lymphocytes and of killer cell self-protection. Immunol. Rev. **103**:87-98.

Munger, W.E., Berrebi, G.A., Henkart, P.A. (1988). Possible involvement of CTL granule proteases in target cell DNA breakdown. *Immunol. Rev.* **103**:99-109.

Murre, C., McCaw, P.S. and Baltimore, D. (1989). A new DNA binding and dimerization motif in immunoglobulin enhancer binding, *daughterless*, MyoD, and myc proteins. *Cell* **56**:777-783.

Nabholz, M. and MacDonald, H.R. (1983). Cytolytic T lymphocytes. *Ann. Rev. Immunol.* 1:273-306.

Nabholz, M. and Tschopp, J. (1989). CTL-mediated cytolysis: perforin and alternative pathways? *Immunol. Lett.* **20**:179-180.

Nagler-Anderson, C., Verret, C.R., Firmenich, A.A., Berne, M. and Eisen, H.N. (1988). Resistance of primary $CD8^+$ cytotoxic T lymphocytes to lysis by cytotoxic granules from cloned T cell lines. *J. Immunol.* **141**:3299-3305.

Naranjo, J.R., Mellström, B., Auwerx, J., Mollinedo, F. and Sassone-Corsi, P. (1990). Unusual c-*fos* induction upon chromaffin PC12 differentiation by sodium butyrate: loss of fos autoregulatory function. *Nucleic Acids Res.* **18**:3605-3610.

Neckers, L.M. and Cossman, J. (1983). Transferrin receptor induction in mitogen-stimulated human T lymphocytes is required for DNA synthesis and cell division and is regulated by interleukin 2. *Proc. Natl. Acad. Sci. USA* **80**:3494-3498.

Norment, A.M., Salter, R.D., Parham, P., Engerhard, V.H. and Littman, D.R. (1988). Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature* **336**:79-81.

Novak. T.J., White, P.M. and Rothenberg, E.V. (1990). Regulatory anatomy of the murine interleukin-2 gene. *Nucleic Acids Res.* **18**:4523-4533.

Odake, S., Kam, C.-M., Narasimhan, L., Poe, M., Blake, J.T., Krahenbühl, O., Tschopp, J. and Powers, J.C. (1991). Human and murine cytotoxic T lymphocyte serine proteases: subsite mapping with peptide thioester substrates and inhibition of enzyme activity and cytolysis by isocoumarins. *Biochemistry* **30**:2217-2227.

Oliva, R., Bazett-Jones, D.P., Locklear, L. and Dixon, G.H. (1990). Histone hyperacetylation can induce unfolding of the nucleosome core particle. *Nucleic Acids Res.* **18**:2739-2747.

Ostergaard, H.L. and Clark, W.R. (1989). Evidence for multiple lytic pathways used by cytotoxic T lymphocytes. *J. Immunol.* **143**:2120-2126.

Ostergaard, H.L., Kane, K.P., Mescher, M.F. and Clark., W.R. (1987). Cytotoxic T lymphocyte mediated lysis without release of serine esterase. *Nature* **330**:71-74.

Paciucci, R. and Pellicer, A. (1991). Dissection of the mouse N-ras gene upstream regulatory sequences and identification of the promoter and a negative regulatory element. *Mol. Cell. Biol.* **11**:1334-1343.

Pardoll, D.M., Fowlkes, B.J., Bluestone, J.A., Kruisbeek, A.M., Maloy, W.L., Coligan, J.E. and Schwartz, R.H. (1987). Differential expression of two distinct T cell receptors during thymocyte development. *Nature* **326**:79-81.

Partington, G.A., Yarwood, N.J. and Rutherford, T.R. (1984). Human globin gene transcription in injected *Xenopus* oocytes: enhancement by sodium butyrate *EMBO J.* **3**:2787-2792.

Pasternack, M.S. (1988). Cytotoxic T-lymphocytes. Adv. Intern. Med. **33**:17-44.

Pasternack, M.S. and Eisen, H.N. (1985). A novel serine esterase expressed by cytotoxic T lymphocytes. *Nature* **314**:743-745.

Pauza, C.D. (1987). Regulation of human T-lymphocyte gene expression by interleukin 2: immediate-response genes include the proto-oncogene c-myb. *Mol. Cell. Biol.* **7**:342-348.

Payvar, F., DeFranco, D., Firestone, G.L., Edgar, B., Wrange, Ö., Okret, S., Gustafsson, J.-A. and Yamamoto, K.R. (1983). Sequence-specific binding of glucocorticoid receptor to MTV DNA at sites within and upstream of the transcribed region. *Cell* **35**:381-392.

Pei, D. and Shih, C. (1991). An "attenuator domain" is sandwiched by two distinct transactivation domains in the transcription factor C/EBP. *Mol. Cell. Biol.* **11**:1480-1487.

Pemberton, R.M., Wraith, D.C. and Askonas, B.A. (1990). Influenza peptide-induced self-lysis and down-regulation of cloned cytotoxic T cells. *Immunology* **70**:223-229.

Pereira. H.A., Spitznagel, J.K., Pohl, J., Wilson, D.E., Morgan, J., Palings, I. and Larrick, J.W. (1990). CAP37, a 37 kD human neutrophil granule cationic protein shares homology with inflammatory proteinases. *Life Sciences* **46**:189-196.

Perrine, S.P., Miller, B.A., Faller, D.V., Cohen, R.A., Vichinsky, E.P., Hurst, D., Lubin, B.H. and Papayannopoulou, T. (1989). Sodium butyrate enhances fetal globin gene expression in erythroid progenitors of patients with Hb SS and β thalassemia. *Blood* **74**:454-459.

Persson, H. and Leder, P. (1984). Nuclear localization and DNA binding properties of a protein expressed by human c-myc oncogene. *Science* **225**:718-721.

Podack, E.R. (1985). Molecular mechanism of lymphocyte-mediated tumor cell lysis. *Immunol. Today* **6**:21-27.

Podack, E.R. and Dennert, G. (1983). Cell-mediated cytolysis: assembly of two types of tubules with putative cytolytic function by cloned natural killer cells. *Nature* **302**:442-445.

Pomerantz, J.L., Mauxion, F., Yoshida, M., Greene, W.C., Sen, R. (1989). A second sequence element located 3' to the NF-κB-binding site regulates IL-2 receptor-α gene induction. *J. Immunol.* **143**:4275-4281.

Pongubala, J.M.R. and Atchison, M.L. (1991). Functional characterization of the developmentally controlled immunoglobulin κ 3' enhancer: regulation by Id, a repressor of helix-loop-helix transcription factors. *Mol. Cell. Biol.* **11**:1040-1047.

Prendergast, J.A., Pinkoski, M., Wolfenden, A. and Bleackley, R.C. (1991). Structure and evolution of the cytotoxic cell proteinase genes CCP3, CCP4 and CCP5. *J. Mol. Biol.* **220**:867-875.

Proudfoot, N. (1991). Poly(A) signals. *Cell* **64**:671-674.

Queen, C. and Stafford, J. (1984). Fine mapping of an immunoglobulin gene activator. *Mol. Cell. Biol.* **4**:1042-1049.

Rabellino, E.M., Ross, G.D. and Polley, M. J. (1978). Membrane receptors of mouse leukocytes. I. Two types of complement receptors for different regions of C3. *J. Immunol.* **120**:871-879.

Ransone, L.J., Wamsley, P., Morley, K.L. and Verma, I.M. (1990). Domain swapping reveals the modular nature of fos, jun, and CREB proteins. *Mol. Cell. Biol.* **10**:4565-4573.

Ratanavongsiri, J., Igarashi, S., Mangal, S., Kilgannon, P., Fu, A. and Fotedar, A. (1990). Transcription of the T cell receptor β-chain gene is controlled by multiple regulatory elements. *J. Immunol.* **144**:1111-1119.

Redmond, M.J., Letellier, M., Parker, J.M.R., Lobe, C.G., Havele, C., Paetkau, V. and Bleackley, R.C. (1987). A serine protease (CCP1) is sequestered in the cytoplasmic granules of cytotoxic T lymphocytes. *J. Immunol.* **139**:3184-3188.

Reeves, R. (1984). Transcriptionally active chromatin. *Biochim. Biophys. Acta* **782**:343-393.

Renkawitz, R., Schütz, G., von der Ahe, D. and Beato, M. (1984). Sequences in the promoter region of the chicken lysozyme gene required for steroid regulation and receptor binding. *Cell* **37**:503-510.

Rhodes, D. and Klug, A. (1986). An underlying repeat in some transcriptional control sequences corresponding to half a double helical turn of DNA. *Cell* **46**:123-132.

Ridsdale, J.A., Hendzel, M.J., Delcuve, G.P. and Davie, J.R. (1990). Histone acetylation alters the capacity of the H1 histones to condense transcriptionally active/competent chromatin. *J. Biol. Chem.* **265**:5150-5156.

Riggs, K.J., Merrell, K.T., Wilson, G. and Calame, K. (1991). Common factor 1 is a transcriptional activator which binds in the c-myc promoter, the skeletal α-actin promoter, and the immunoglobulin heavy-chain enhancer. *Mol. Cell. Biol.* **11**:1765-1769.

Riis, B., Rattan, S.I.S., Clark, B.F.C. and Merrick, W.C. (1990). Eukaryotic protein elongation factors. *Trends Biochem. Sci.* **15**:420-424.

Ringold, G.M., Dieckmann, B., Vannice, J.L., Trahey, M. and McCormick, F. (1984). Inhibition of protein synthesis stimulates the transcription of human β-interferon genes in Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA* **81**:3964-3968.

Rius, C., Cabanas, C. and Aller, P. (1990). The induction of vimentin gene expression by sodium butyrate in human promonocytic leukemia U937 cells. *Exp. Cell Res.* **188**:129-134.

Rivera, V.M., Sheng, M. and Greenberg, M.E. (1990). The inner core of the serum response element mediates both the rapid induction and subsequent repression of c-fos transcription following serum stimulation. *Genes Dev.* **4**:255-268.

Rosenberg, S.A. (1988). Immunotherapy of cancer using interleukin 2: current status and future prospects. *Immunol. Today* **9**:58-62.

Rossi, P. and de Crombrugghe, B. (1987). Identification of a cell-specific transcriptional enhancer in the first intron of the mouse α2 (type I) collagen gene. *Proc. Natl. Acad. Sci. USA* **84**:5590-5594.

Rudd, C.E., Trevillyan, J.M., Dasgupta, J.D., Wong, L.L. and Schlossman, S.F. (1988). The CD4 receptor is complexed in detergent lysates to a protein-tyrosine kinase (pp58) from human T lymphocytes. *Proc. Natl. Acad. Sci. USA* **85**:5190-5194.

Russell, J.H. (1983). Internal disintegration model of cytotoxic lymphocyte-induced target damage. *Immunol. Rev.* **72**:97-118.

Russell, J.H. and Dobos, C.B. (1980). Mechanisms of immune lysis. II. CTL-induced nuclear disintegration of the target begins within minutes of cell contact. *J. Immunol.* **125**:1256-1261.

Russell, J.H., Masakowski, V.R. and Dobos, C.B. (1980). Mechanisms of immune lysis. I. Physiological distinction between target cell death mediated by cytotoxic T lymphocytes and antibody plus complement. *J. Immunol.* **124**:1100-1105.

Saltzman, E.M., White, K. and Casnellie, J.E. (1990). Stimulation of the antigen and interleukin-2 receptors on T lymphocytes activates distinct tyrosine protein kinases. *J. Biol. Chem.* **265**:10138-10142.

Samelson, L.E., Maitray, P.D., Weissman, A.M., Harford, J.B. and Klausner, R.D. (1986). Antigen activation of murine T cells induces tyrosine phosphorylation of a polypeptide associated with the T cell antigen receptor. *Cell* **46**:1083-1090.

Sassone-Corsi, P. and Verma, I.M. (1987). Modulation of c-fos gene transcription by negative and positive cellular factors. *Nature* **326**:507-510.

Sauerwald, A., Hoesche, C., Oschwald, R. and Kilimann, M.W. (1990). The 5'-flanking region of the synapsin I gene. *J. Biol. Chem.* **265**:14932-14937.

Savagner, P., Miyashita, T. and Yamada, Y. (1990). Two silencers regulate the tissue-specific expression of the collagen II gene. *J. Biol. Chem.* **265**:6669-6674.

Sawadogo, M. and Sentenac, A. (1990). RNA polymerase B (II) and general transcription factors. *Ann. Rev. Biochem.* **59**:711-754.

Scafe, C., Chao, D., Lopes, J., Hirsch, J.P., Henry, S. and Young, R.A. (1990). RNA polymerase II C-terminal repeat influences response to transcriptional enhancer signals. *Nature* **347**:491-494.

Schaffner, W. (1989). How do different transcription factors binding the same DNA sequence sort out their jobs? *Trends Genet.* **5**:37-39.

Schibler, U. and Sierra, F. (1987). Alternative promoters in developmental gene expression. *Ann. Rev. Genet.* **21**:237-257.

Schmid, J. and Weissman, C. (1987). Induction of mRNA for a serine protease and a β-thromboglobulin-like protein in mitogen-stimulated human leukocytes. *J. Immunol.* **139**:250-256.

Schmidt, R., Cathelineau, C., Cavey, M.T., Dionisius, V., Michel, S., Shroot, B. and Reichert, U. (1989). Sodium butyrate selectively antagonizes the inhibitory effect of retinoids on cornified envelope formation in cultured human keratinocytes. *J. Cell. Physiol.* **140**:281-287.

Schmidt, R.E., MacDermott, R.P., Bartley, G., Bertovich, M., Amato, D.A., Austen, K.F., Schlossman, S.F., Stevens, R.L. and Ritz, J. (1985). Specific release of proteoglycans from human natural killer cells during target lysis. *Nature* **318**:289-291.

Scollay, R.G., Butcher, E.C. and Weissman, I.L. (1980). Thymus cell migration. Quantitative aspects of cellular traffic from the thymus to the periphery in mice. *Eur. J. Immunol.* **10**:210-218.

Scollay, R., Reichert, R., Kyewski, B., Fink, P., Shortman, K., Ezine, S., Gallatin, M., Butcher, E., Rouse, R., Kaplan, H. and Weissman, I. (1984). Thymic lymphocyte maturation. In *The lymphocyte: Structure and Function.* (J.J. Marchalonis, ed.), New York: Marcel Dekker, pp. 179-248.

Sealy, L. and Chalkley, R. (1978). The effect of sodium butyrate on histone modification. *Cell* **14**:115-121.

Sehgal, A., Patil, N. and Chao, M. (1988). A constitutive promoter directs expression of the nerve growth factor receptor gene. *Mol. Cell. Biol.* **8**:3160-3167.

Serafin, W.E., Katz, H.R., Austen, K.F. and Stevens, R.L. (1986). Complexes of heparin proteoglycans, chondroitin sulfate E proteoglycans, and [^{3}H]diisopropyl fluorophosphate-binding proteins are exocytosed from activated mouse bone marrow-derived mast cells. *J. Biol. Chem.* **261**:15017-15021.

Serafin, W.E., Reynolds, D.S., Rogelj, S., Lane, W.S., Conder, G.A., Johnson, S.S., Austen, K.F. and Stevens, R.L. (1990). Identification and molecular cloning of a novel mouse mucosal mast cell serine protease. *J. Biol. Chem.* **265**:423-429.

Serafin, W.E., Sullivan, T.P., Conder, G.A., Ebrahimi, A., Marcham, P., Johnson, S.S., Austen, K.F. and Reynolds, D.S. (1991). Cloning of the cDNA and gene for mouse mast cell protease 4. *J. Biol. Chem.* **266**:1934-1941.

Serfling, E., Jasin, M. and Schaffner, N. (1985). Enhancers and eukaryotic gene transcription. *Trends Genet.* **1**:224-230.

Shinkai, Y., Takio, K. and Okumura, K. (1988). Homology of perforin to the ninth component of complement (C9). *Nature* **334**:525-527.

Shinkai, Y., Yoshida, M.C., Maeda, K., Kobata, T., Maruyama, K., Yodoi, J., Yagita, H. and Okumura, K. (1989). Molecular cloning and chromosomal assignment of a human perforin (PFP) gene. *Immunogenetics* **30**:452-457.

Shyu, A.-B., Belasco, J.G. and Greenberg, M.E. (1991). Two distinct destabilizing elements in the c-fos message trigger deadenylation as a first step in rapid mRNA decay. *Genes Dev.* **5**:221-231.

Siebenlist, U., Durand, D.B., Bressler, P., Holdbrook, N.J., Norris, C.A., Kamoun, M., Kant, J.A. and Crabtree, G.R. (1986). Promoter region of interleukin-2 gene undergoes chromatin structure changes and confers inducibility on chloramphenicol acetyltransferase gene during activation of T cells. *Mol. Cell. Biol.* **6**:3042-3049.

Simon, M.M., Fruth, U., Simon, H.G. and Kramer, M.D. (1987). Evidence for the involvement of a T-cell-associated serine protease (TSP-1) in cell killing. *Annls Inst. Pasteur Immunol.* **138**:309-314.

Simon, M.M., Simon, H.G., Fruth, U., Epplen, J., Muller Hermelink, H.K. and Kramer, M.D. (1987). Cloned cytolytic T effector cells and their malignant variants produce an extracellular matrix degrading trypsin-like serine proteinase. *Immunology* **60**:219-230.

Sleckman, B.P., Peterson, A., Jones, W.K., Foran, J.A., Greenstein, J.L., Seed, B. and Bukaroff, S.J. (1987). Expression and function of CD4 in a murine T-cell hybridoma. *Nature* **328**:351-353.

Smith, C.W.J., Patton, J.G. and Nadal-Ginard, B. (1989). Alternative splicing in the control of gene expression. *Ann. Rev. Genet.* **23**:527-577.

Smith, M.R. and Greene, W.C. (1989). The same 50-kDa cellular protein binds to the negative regulatory elements of the interleukin 2 receptor α-chain gene and the human immunodeficiency virus type 1 long terminal repeat. *Proc. Natl. Acad. Sci. USA* **86**:8526-8530.

Spencer, D.M., Hsiang, Y.-H., Goldman, J.P. and Raulet, D.H. (1991). Identification of a T cell-specific transcriptional enhancer located 3' of Cγ1 in the murine T cell receptor γ locus. *Proc. Natl. Acad. Sci. USA* **88**:800-804.

Spits, H., van Schooten, W., Keizer, H., van Seventer, G., van DeRijn, M., Terhorst, C. and DeVries, J.E. (1986). Alloantigen recognition is preceded by nonspecific adhesion of cytotoxic T cells and target cells. *Science* **232**:403-405.

Spitzner, J.R., Chung, I.K. and Muller, M.T. (1989). Eukaryotic topoisomerase II preferentially cleaves alternating purine-pyrimidine repeats. *Nucleic Acids Res.* **18**:1-11.

Springer, T.A., Dustin, M.L., Kishimoto, T.K. and Marlin, S.D. (1987). The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. *Ann. Rev. Immunol.* **5**:223-252.

Stenlund, A. and Botchan, M.R. (1990). The E2 trans-activator can act as a repressor by interfering with a cellular transcription factor. *Genes Dev.* **4**:123-136.

Stern, J.B. and Smith, K.A. (1986). Interleukin-2 induction of T-cell G1 progression and c-myb expression. *Science* **233**:203-206.

Stoddart, J.H., Lane, M.A. and Niles, R.M. (1989). Sodium butyrate suppresses the transforming activity of an activated N-ras oncogene in human colon carcinoma cells. *Exp. Cell Res.* **184**:16-27.

Strominger, J.L. (1989). Developmental biology of T-cell receptors. *Science* **244**:943-950.

Struhl, K. (1989). Molecular mechanisms of transcriptional regulation in yeast. *Ann. Rev. Biochem.* **58**:1051-1077.

Tang, D.-C. and Taylor, M.W. (1990). Transcriptional activation of the adenine phosphoribosyltransferase promoter by an upstream butyrate-induced Moloney murine sarcoma virus enhancer-promoter element. *J. Virol.* **64**:2907-2911.

Tang, S.-J., Ko, L.-W., Lee, Y.-H. and Wang, F.-F. (1990). Induction of *fos* and *sis* proto-oncogenes and genes of the extracellular matrix proteins during butyrate induced glioma differentiation. *Biochim. Biophys. Acta* **1048**:59-65.

Terhorst, C., Alarcon, B., DeVries, J. and Spits, H. (1988). T lymphocyte recognition and activation. In *Molecular Immunology*. (B.D. Hames and D.M. Glover, eds). IRL Press, Washington. pp. 145-188.

Tirosh, R. and Berke, G. (1985). T-lymphocyte-mediated cytolysis as an excitatory process of the target. *Cell. Immunol.* **95**:113-123.

Tonegawa, S. (1983). Somatic generation of antibody diversity. *Nature* **302**:575-581.

Toscani, A., Robert Soprano, D. and Soprano, K.J. (1990). Sodium butyrate in combination with insulin or dexamethasone can terminally differentiate actively proliferating Swiss 3T3 cells into adipocytes. *J. Biol. Chem.* **265**:5722-5730.

Trenn, G., Takayama, H. and Sitkovsky, M.V. (1987). Exocytosis of cytolytic granules may not be required for target cell lysis by cytotoxic T lymphocytes. *Nature* **330**:72-74.

Trinchieri, G. (1989). Biology of natural killer cells. *Adv. Immunol.* **47**:187-309.

Tschopp, J. and Masson, D. (1987). Inhibition of the lytic activity of perforin (cytolysin) and of late complement components by proteoglycans. *Mol. Immunol.* **24**:907-913.

Tschopp, J. and Nabholz, M. (1987). The role of cytoplasmic granule components in cytolytic lymphocyte-mediated cytolysis. *Annls. Inst. Pasteur Immunol.* **138**:290-295.

Tschopp, J. and Nabholz, M. (1990). Perforin-mediated target cell lysis by cytolytic T lymphocytes. *Ann. Rev. Immunol.* **8**:279-302.

Tunnacliffe, A., Sims, J.E. and Rabbitts, T.H. (1986). T3δ pre-mRNA is transcribed from a non-TATA promoter and is alternatively spliced in human T cells. *EMBO J.* **5**:1245-1252.

Ullman, K.S., Northrop, J.P., Verweij, C.L. and Crabtree, G.R. (1990). Transmission of signals from the T lymphocyte antigen receptor to the genes responsible for cell proliferation and immune function: the missing link. *Ann. Rev. Immunol.* **8**:421-452.

Umek, R.M., Friedman, A.D. and McKnight, S.L. (1991). CCAAT-enhancer binding protein: a component of a differentiation switch. *Science* **251**:288-292.

Unanue, E.R. (1976). Secretory function of mononuclear phagocytes. Am. J. Pathol. **83**:395-417.

Unanue, E.R. and Allen, P.M. (1987). The basis for the immunoregulatory role of macrophages and other sensory cells. *Science* **236**:551-557.

Unanue, E.R. and Benacerraf, B. (1984). *Textbook of Immunology*. 2nd Edition, Williams and Wilkins, Baltimore. 324 pages.

Unkeless, J.C., Fleit, H. and Mellman, I.S. (1981). Structural aspects and heterogeneity of immunoglobulin Fc receptors. *Adv. Immunol.* **31**:247-270.

Urban, M.B. and Baeuerle, P.A. (1990). The 65-kD subunit of NF-κB is a receptor for IκB and a modulator of DNA-binding specificity. *Genes Dev.* **4**:1975-1984.

Vacher, J. and Tilghman, S. (1990). Dominant negative regulation of the mouse α-fetoprotein gene in adult liver. *Science* **250**:1732-1735.

Valerio, D., Duyvesteyn, M.G.C., Dekker, B.M.M., Weeda, G., Berkvens, T.M., van der Voorn, L., van Ormondt, H. and van der Eb, A.J. (1985). Adenosine deaminase: characterization and expression of a gene with a remarkable promoter. *EMBO J.* **4**:437-443.

Vallee, B.L., Coleman, J.E. and Auld, D.S. (1991). Zinc fingers, zinc clusters, and zinc twists in DNA-binding protein domains. *Proc. Natl. Acad. Sci. USA* **88**:999-1003.

Vanderslice, P., Ballinger, S.M., Tam, E.K., Goldstein, S.M., Craik, C.S. and Caughey, G.H. (1990). Human mast cell tryptase: multiple cDNAs and genes reveal a multigene serine protease family. *Proc. Natl. Acad. Sci. USA* **87**:3811-3815.

Veillette, A., Bookman, M.A., Horak, E.M., Bolen, J.B. (1988). The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase $p56^{lck}$. *Cell* **55**:301-308.

Velotti, F., Palmieri, G., Morrone, S., Piccoli, M., Frati, L. and Santoni, A. (1989). Granzyme A expression by normal rat natural killer (NK) cells *in vivo* and by interleukin 2-activated NK cells *in vitro*. *Eur. J. Immunol.* **19**:575-578.

Verret, C.R., Firmenich, A.A., Kranz, D.M. and Eisen, H.N. (1987). Resistance of cytotoxic T lymphocytes to the lytic effects of their toxic granules. *J. Exp. Med.* **166**:1536-1547.

Vinson, C.R., Sigler, P.B. and McKnight, S.L. (1989). Scissors-grip model for DNA recognition by a family of leucine zipper proteins. *Science* **246**:911-916.

von Boehmer, H. (1988). The developmental biology of T lymphocytes. *Ann. Rev. Immunol.* **6**:309-326.

von Kries, J.P., Buhrmester, H. and Strätling, W.H. (1991). A matrix/scaffold attachment region binding protein: identification, purification, and mode of binding. *Cell* **64**: 123-135.

Walden, P.R. and Eisen, H.N. (1990). Cognate peptides induce self-destruction of $CD8^+$ cytolytic T lymphocytes. *Proc. Natl. Acad. Sci. USA* **87**:9015-9019.

Waldmann, T.A. (1991). The interleukin-2 receptor. *J. Biol. Chem.* **266**:2681-2684.

Wang, J., Oketani, M. and Watanabe, T. (1991). Positive and negative regulation of immunoglobulin gene expression by a novel B-cell-specific enhancer element. *Mol. Cell. Biol.* **11**:75-83.

Warner, N.L. (1974). Membrane immunoglobulins and antigen receptors on B and T lymphocytes. *Adv. Immunol.* **19**:67-216.

Weiher, H., König, M. and Gruss, P. (1983). Multiple point mutations affecting the Simian virus 40 enhancer. *Science* **219**:626-631.

Weintraub, H. (1984). Histone H1-dependent chromatin superstructures and the suppression of gene activity. *Cell* **38**:17-27.

Weiss, S.J. (1989). Tissue destruction by neutrophils. *N. Engl. J. Med.* **320**:365-376.

Wickens, M.P. and Dahlberg, J.E. (1987). RNA-protein interactions. *Cell* **51**:339-342.

Widom, R.L., Ladias, J.A.A., Kouidou, S. and Karathanasis, S.K. (1991). Synergistic interactions between transcription factors control expression of the apolipoprotein AI gene in liver cells. *Mol. Cell. Biol.* **11**:677-687.

Wilde, C.G., Snable, J.L., Griffith, J.E. and Scott, R.W. (1990). Characterization of two azurophil granule proteases with active-site homology to neutrophil elastase. *J. Biol. Chem.* **265**:2038-2041.

Winoto, A. and Baltimore, D. (1989a). αβ lineage-specific expression of the α T cell receptor gene by nearby silencers. *Cell* **59**:649-655.

Winoto, A. and Baltimore, D. (1989b). A novel, inducible and T cell-specific enhancer located at the 3' end of the T cell receptor α locus. *EMBO J.* **8**:729-733.

Wirth, T., Priess, A., Annweiler, A., Zwilling, S. and Oeler, B. (1991). Multiple Oct2 isoforms are generated by alternative splicing. *Nucleic Acids Res.* **19**:43-51.

Wisdom, R. and Lee, W. (1991). The protein-coding region of c-myc mRNA contains a sequence that specifies rapid mRNA turnover and induction by protein synthesis inhibitors. *Genes Dev.* **5**:232-243.

Wright, S.C. and Bonavida, B. (1982). Studies on the mechanism of natural killer (NK) cell-mediated cytotoxicity (CMC). I. Release of cytotoxic factors specific for NK-sensitive target cells (NKCF) during co-culture of NK effector cells with NK target cells. *J. Immunol.* **129**:433-439.

Wright, S.C. and Bonavida, B. (1987). Studies on the mechanism of natural killer cell-mediated cytotoxicity. VII: Functional comparison of human natural killer cytotoxic factor with recombinant lymphotoxin and tumor necrosis factor. *J. Immunol.* **138**:1791-1798.

Yamamoto, K.K. (1985). Steroid receptor regulated transcription of specific genes at gene networks. *Ann. Rev. Genet.* **19**:209-252.

Yamamoto, K.K., Gonzalez, G.A., Biggs III, W.H. and Montminy, M.R. (1988). Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. *Nature* **334**:494-498.

Yannelli, J.R., Sullivan, J.A., Mandell, G.L. and Engelhard, V.H. (1986). Reorientation and fusion of cytotoxic T lymphocyte granules after interaction with target cells as determined by high resolution cinemicrography. *J. Immunol.* **136**:377-382.

Young, J.D.-E (1989). Killing of target cells by lymphocytes: a mechanistic view. *Physiol. Rev.* **69**:250-314.

Young, J.D.-E and Liu, C.-C. (1988a). How do cytotoxic T lymphocytes avoid self-lysis? *Immunol. Today* **9**:14-15.

Young, J.D.-E and Liu, C.-C. (1988b). Multiple mechanisms of lymphocyte-mediated killing. *Immunol. Today* **9**:140-144.

Young, L.H.Y., Klavinskis, L.S., Oldstone, M.B.A. and Young, J.D.-E (1989). *In vivo* expression of perforin by CD8$^+$ lymphocytes during an acute viral infection. *J. Exp. Med.* **169**:2159-2171.

Young, L.H.Y., Liu, C.-C., Joag, S., Rafti, S. and Young, J.D.-E (1990). How lymphocytes kill. *Ann. Rev. Med.* **41**:45-54.

Young, L.H.Y., Peterson, L.B., Wicker, L.S., Persechini, P.M., Young, J.D.-E (1989). *In vivo* expression of perforin by CD8$^+$ lymphocytes in autoimmune disease. Studies on spontaneous and adoptively transferred diabetes in nonobese diabetic (NOD) mice. *J. Immunol.* **143**:3994-3999.

Zalman, L.S., Brothers, M.A., Chiu, F.J., Müller-Eberhard, H.J. (1986). Mechanism of cytotoxicity of human large granular lymphocytes: relationship of the cytotoxic lymphocyte protein to the ninth component (C9) of human complement. *Proc. Natl. Acad. Sci. USA* **83**:5262-5266.

Zamore, P.D., Zapp, M.L. and Green, M.R. (1990). RNA binding: βs and basics. *Nature* **348**:485-486.

Zhang, J. and Corden, J.L. (1991). Phosphorylation causes a conformational change in the carboxyl-terminal domain of the mouse RNA polymerase II largest subunit. *J. Biol. Chem.* **266**:2297-2302.

Zychlinsky, A., Zheng, L.M., Liu, C.-C. and Young, J.D.-E (1991). Cytolytic lymphocytes induce both apoptosis and necrosis in target cells. *J. Immunol.* **146**:393-400.

CHAPTER II

FACTORS INFLUENCING TRANSIENT EXPRESSION IN CYTOTOXIC T CELLS FOLLOWING DEAE DEXTRAN-MEDIATED GENE TRANSFER[1]

A. INTRODUCTION

Using reverse genetics it is now possible to alter the phenotype of a cell at will. However, in order to achieve this, it must be possible to introduce the gene of interest into the recipient cells. For many standard cell lines efficient transfection protocols have been worked out. Unfortunately, not all cell types are amenable to these standard regimens.

Cytotoxic T lymphocytes play a major role as effectors of cell mediated immunity. It is certainly appealing to imagine using reverse genetics with these cells in order to gain some therapeutic advantage. However, very little work has been reported on transfection efficiency in this most interesting cell type. In addition, the development of such a method would be a most useful tool to aid in the delineation of the elements that control expression of CTL-specific genes and thus understanding CTL activation.

Successful stable transfection of a variety of T cell lines has been reported using electroporation (helper T cell clone (1), T cell hybridoma (2), thymoma cell lines such as EL4 and BW5147 (3, 4, 5), T cell tumor lines such as Jurkat (6, 7)), protoplast fusion (T lymphoma cell line (8), T cell hybridoma (9), T cell clone (10,11)), calcium phosphate coprecipitation (T cell lymphoma (12), T cell leukemia cells (13)), DEAE dextran (T cell clone (14), peripheral blood lymphocytes (15)) and retroviral vectors (T lymphocytes (11), tumor-infiltrating lymphocytes (16), glioma-specific CTLs (17)). In addition, many investigators have reported significant transitory expression in T cell tumor lines such as Jurkat (18, 19), thymoma cell lines (20, 21), T leukemia cells (22), HIV-1 transformed T cell clones (23) using the DEAE dextran-mediated gene transfer method. Short term transfection of peripheral blood lymphocytes and a few T lymphoma cell lines have been achieved by electroporation (24, 25). There has been one report describing transient expression of a transfected gene in a murine cytotoxic T cell clone (10) and one describing it in a human T leukemia cell line using protoplast fusion (26). Thus, there is a paucity of information on transfection of non-transformed cytolytic T cell clones.

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In an attempt to define the best method for transfection of cytotoxic T cell lines, we tried a number of different protocols such as electroporation (27), lipofection (28), calcium phosphate coprecipitation (29), polybrene-assisted gene transfer (30) and DEAE dextran-mediated transfer (20) and its variants (31,32). The latter protocol gave significant levels of *cat* transcription using pRSVcat as the standard expression vector. We have ascertained the critical parameters to obtain optimal transfection efficiency in an adherent cytotoxic cell line MTL 2.8.2. In addition, we show that the adapted DEAE dextran procedure is sufficiently sensitive to allow detection of *cat* activity driven, not only by strong viral promoters but also and most importantly, by CTL-specific gene promoters. A comparison of the strength of two of our CTL-serine protease promoters to those of other viral promoters (RSV, SV40) and other cellular promoters (fos, tk) is presented.

B. MATERIALS AND METHODS

1. *Cell lines and culture techniques*

The cytotoxic T cell line MTL 2.8.2 was generated from CBA/Balb/c mice as described previously (33). It is an IL2 dependent cell line that can proliferate in the absence of antigen. These cells were cultured in RPMI 1640 medium (Gibco Laboratories, Life Technologies, Inc., Grand Island, NY) supplemented with 10% (v/v) fetal calf serum (Gibco) and 100 µM of β-mercaptoethanol. This is referred to as RHFM medium. MTL 2.8.2 cells were maintained in RHFM containing 30 units/ml of recombinant human IL2.

2. *Plasmids and their preparation*

Plasmids used were obtained from several investigators: pRSVcat (34) and pSV2cat (35) from R. Aubin (Health and Welfare, Canada), pA10cat2 (36) from P. Leder (Harvard), pTKcat from R. Miksicek (Heidelberg) and pFCΔ56 (37) from W. Leonard (National Institute of Health, U.S.A.). The construction of pB10cat and pC11cat was as follows: the 5'-flanking regions of the mouse CTL-serine protease B10 and C11 genes (38) were excised using Pst1/Acc1 and Taq1/AvaII respectively and inserted directly into pGEM1cat at the Pst1/Acc1 restriction sites or indirectly in the Sma1 site of pGEM2cat after polishing the ends with *E. coli* DNA polymerase 1 (large fragment) as described in Davis (39). The pGEMcat vectors were obtained by introducing the BamH1/HindIII *cat*-splice-polyadenylation cartridge of pSV2cat, blunt-ended using *E. coli* Klenow fragment, into the PvuII site of either pGEM1 or pGEM2 plasmids (Promega Corporation). All ligations were done at 14°C for 18 hours as detailed in Davis (39).

Plasmid stocks were propagated in *E. coli* strain DH5α and isolated using the alkaline lysis procedure of Birnboim and Doly (40). With careful manipulations, avoiding mechanical vortexing at any step, we routinely obtained preparations consisting of more than 90% form 1 (covalently closed circular, supercoiled) DNA molecules, free of contaminating bacterial chromosomal DNA, RNA and other host cell components. This transfection grade DNA ensured both consistency and optimal levels of gene expression in transient assays.

3. *Transfection protocols*

Based on the DEAE dextran gene transfer technique of Fujita (20) and taking the modifications of Gopal (31) and Sussman and Milman (32) into consideration, we have adapted the dextran method for cytotoxic T cells as follows: two days prior to gene transfer, mid-log MTL 2.8.2 cells were seeded at 4 x 10^4 cells/ml or 1 x 10^6 cells/75 cm^2 flask in RHFM supplemented with 30 units/ml of recombinant human IL2. Cells were then collected using PBS/EDTA (20 mM). The density at harvest was 3.75 to 4 x 10^6 cells/flask. The cell pellet was washed twice with serum free medium and 1.25 x 10^7 cells per sample were dispensed in 17 x 100 mm polypropylene tubes (Fisher). Each cell pellet was resuspended in 1 ml of transfection buffer (0.5 ml of TBS: 25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.5 ml of serum free medium) to which 500 µg/ml of DEAE dextran was added (stock: 100 µg/µl made in dH_2O and filter sterilized) then 10 µg of appropriate plasmid form 1 DNA. The samples were mixed gently. DNA adsorption was set at room temperature in the laminar flow hood for 15 minutes. The cells were then rinsed with 10 ml of serum free medium and spun at 1200 rpm for 5 minutes at room temperature. This washing step was repeated one more time. Cells were finally resuspended in 3 ml of RHFM + IL2 (30 units/ml). Each sample was dispensed in three 100 mm dishes containing 11 ml of complete medium RHFM + IL2 + 7.5 mM sodium butyrate (Sigma, made up as a 500 mM stock in serum free RH medium, filter sterilized) and were incubated for 12 hours at 37°C in 5% CO_2 atmosphere. The medium was then gently aspirated, each culture was fed fresh growth medium and was incubated for a further 36 hours before harvest.

Whenever stated the permeabilization with DMSO was performed as follows: immediately after adsorption, DMSO (Spectranalysed, UV cutoff at 262 nm; Fisher Scientific Co.) was added dropwise to the cells to achieve the desired final concentration (v/v). Special care was given to mix the solution between each drop addition. The cells were left in the laminar flow hood (room temperature) or placed in a 37°C/5% CO_2 incubator for the indicated lengths of time. The cells were then washed as indicated earlier and resuspended in 3 ml of complete medium before being dispensed into three 100 mm plates containing 11 ml of complete medium.

For the treatment with chloroquine diphosphate, MTL2.8.2 cells were transfected with 10 µg of pRSVcat DNA using 500 µg/ml of DEAE dextran for 15 minutes at room temperature. They were then washed as indicated earlier and finally resuspended in 3 ml of complete medium i.e. RHFM + IL2 (30 units/ml) supplemented with 25, 50, 75 or 100 µM of chloroquine diphosphate (Sigma, prepared as a 5 mM stock in serum free RH medium) and left at 37°C in a 5% CO_2 incubator for 1, 2, 3 or 4 hours. Cells were then washed twice with fresh medium then resuspended in complete medium and incubated for 48 hours before collection.

For optimization of the sodium butyrate concentration, MTL2.8.2 cells were transfected as described above and the final cell pellets were resuspended in 3 ml of complete medium supplemented with 1, 2, 3, 4, 5, 7.5 or 10 mM sodium butyrate (Sigma, made up as a 500 mM stock in serum free RH medium, filter sterilized) and left at 37°C in a 5% CO_2 incubator for 4 hours (in 17x100 mm tubes) or 12 hours (in 100 mm plates). Cells were then collected and washed twice with fresh medium then resuspended in complete medium and left at 37°C in a 5% CO_2 atmosphere for 48 hours or 36 hours before harvest.

4. *Cat enzyme assay*

MTL 2.8.2 cells were processed for *cat* assays as follows: dishes were washed once with PBS to remove dead cells (floaters) and cells were harvested using PBS/EDTA. Each cell pellet was resuspended in 1 ml PBS and collected by centrifugation (12,000 g for 2 minutes at room temperature) in a 1.5 ml capacity microcentrifuge tube. Samples were usually snap frozen on dry ice and kept at -20°C.

Crude extracts were prepared in 0.1 ml or 0.15 ml of 0.25 M Tris-HCl pH 7.8 + 0.5 mM PMSF (phenylmethylsulphonylfluoride) by subjecting cell pellets to three cycles of freezing on dry ice for 5 minutes and thawing in a 37°C water bath for 5 minutes. Extraction of the soluble protein fraction containing the *cat* enzyme was facilitated by vortexing the broken cells for 30 seconds after each cycle. In addition, broken cells were subjected to 20 strokes of pellet pestle (BDH). Following removal of the cell debris by centrifugation (12000 g for 5 minutes at 4°C), the concentration of protein in the lysates was determined using the Bio-Rad protein assay. *Cat* enzymatic activity was determined based on a protocol described by Gorman *et al.* (35). Samples for a particular series of conditions were always transfected at the same time using the same DNA preparations. An equal amount of protein (usually between 100 to 200 μg) from each culture was incubated with 0.014 μCi of 45 mCi/mmol, 0.1 mCi/ml [^{14}C]chloramphenicol (DuPont/NEN products), 4 mM acetyl coenzyme A (Pharmacia) and 0.5 M Tris-HCl pH 7.8 in a final volume of 150 μl at 37°C for 4 hours to obtain sufficient acetylation to be easily quantitated and still be in the linear portion of the kinetics of chloramphenicol acetylation. Reactions were extracted with 1 ml of ethyl acetate. Extracts were dried under heat and vacuum for 30 minutes, suspended in 25 μl of ethyl acetate, spotted on thin-layer chromatography and developed in a solution of 95% chloroform /5% methanol.

Reaction products were visualized by autoradiography at room temperature and the radioactivity in acetylated and unacetylated spots was quantified by liquid scintillation counting. Results are given in percent conversion of chloramphenicol to its monoacetylated forms. Values reported here represent the mean of three or more independent transfections.

5. *Dot blot analysis*

Low molecular weight DNA was recovered from transfected MTL 2.8.2 cells following the Hirt procedure (41). Dot blot analysis was carried out on DNA from Hirt extracts fixed to nylon membranes (Hybond-N, Amersham Ltd.) by filtration through a multiwell minifold (Schleicher & Schuell, Inc.) as recommended by Amersham Ltd. (Technical Bulletin P1/162/85/1). Hybridization of the blots was performed at 42°C under stringent conditions (50% formamide, 5X SSC (1X SSC: 15 mM sodium citrate, 150 mM NaCl, pH 7.0), 5X Denhardt's solution, 0.1% SDS, 50 mM phosphate buffer pH 6.5, 1 mM $NaPyPO_4$, 100 μM ATP, 2.5 mM EDTA) to [^{32}P]nick-translated pRSVcat plasmid (1 x 10^8 cpm/μg). Membranes were washed under high stringency in 0.1X SSC/0.1% SDS at 56°C and exposed to Kodak XAR-5 film for one day at room temperature.

6. *Cell viability*

As an indicator of cell viability at 48 hours post-transfection (time at which cells were collected for transient *cat* gene expression assays) the cells were washed with PBS once and checked for their ability to exclude the eosin dye using phase contrast microscopy. Enumeration was done with a hemocytometer.

C. RESULTS AND DISCUSSION

1. *Narrowing down the transfection strategies*

There have been very few reports on transient gene transfer in cytotoxic T lymphocytes and our preliminary experiments using a number of standard transfection protocols with the cytotoxic cell line MTL 2.8.2 indicated that it would be problematic. The results summarized in Table II.1 represent the levels of *cat* activity obtained with pRSVcat introduced using standard conditions for the various gene transfer strategies tested. MTL 2.8.2 cells were electroporated following the protocol described by Potter *et al.* (27). The BRL Cell-Porator^{TM} Electroporation System was used to deliver the electric pulses (400-1000 V/cm). Various conditions were also tried following the BRL booklet of guidelines for electroporation, i.e. varying pulse length, field strength, electroporation buffer, temperature at which electroporation is performed, length of recovery in the electroporation buffer after transfection. Lipofection of the same cells was attempted using the BRL Lipofection kit and their guidelines based on Felgner *et al.* (28). Since no basic protocol was available for T cells or B cells at the time, a number of conditions were tried varying the concentration of lipofectin, DNA, cell density, temperature at which adsorption was taking place and length of adsorption period. Attempted transfection of MTL 2.8.2 with polybrene was adapted from Aubin *et al.* (30). Basically,various conditions suggested for fibroblasts were tested and manipulations were performed in tubes rather than in plates to accommodate suspended cell cultures. The conditions used for calcium

DNA-mediated gene transfer methodologies	*Cat* activity chloramphenicol acetylation (%)	Standard deviation (%)	Cell viability (%)
DEAE dextran	30	8	92
Lipofection	0.2	20	43
Electroporation	<0.1	25	20
Calcium phosphate coprecipitation	3	23	60
Polybrene	<0.1	15	20

TABLE II.1 Comparison of transfection efficiencies in MTL2.8.2 cells using various gene transfer strategies. MTL2.8.2 cytotoxic cells were transfected following various established protocols (see details in Results and Discussion section). The absolute values of *cat* activity shown represent the mean of values obtained under the best conditions found during the pilot experiments for each technique. For each protocol 10 µg of supercoiled plasmid DNA was used and adsorbed to cells with the appropriate agents. After washing, the cells were resuspended at the starting density and incubated at 37°C for 40 hours: (1) 50 µg Lipofectin™ Reagent, 3.5 ml cells at 10^6 cells/ml in 60 mm dishes for 5 hours at 37°C; (2) in a BRL Cell-Porator™ with 6 x 10^6 cells in 1 ml of 21 mM Hepes pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose, set at 1000 V/cm, 400 V, 330 µF for 7.7 msec in ice/water; (3) coprecipitated with 12 µg salmon sperm DNA by calcium phosphate on 2 x 10^6 cells per 100 mm dish in 6 ml for 4 hours at 37°C, shocked with 10% DMSO at 37°C for 4.5 min; (4) 10 µg polybrene, 2 ml cells at 10^7 cells/ml in 15 ml polypropylene tubes for 20 hours at 37°C; followed by a shock in 15% DMSO for 4.5 min. The standard deviations are given as a degree of reproducibility between triplicate experiments performed. Cell viability was assessed at time when cells were collected for *cat* determination.

phosphate coprecipitation were those of Wallich *et al.* (42) and Gilman *et al.* (37). Different permeabilization conditions were tried including shocking the cells with DMSO (8 to 28%) or glycerol (15 to 25%) for various lengths of time at room temperature or 37°C. The basic DEAE dextran-assisted gene transfer was that of Fujita *et al.* (20).

In our hands only the DEAE dextran assisted gene transfer allowed us to detect significant *cat* activity in a non-transformed CTL line. The other methods gave very low reproducibility and/or high cytotoxicity or low viability under the wide range of conditions that were tried (see Table II.1). Lipofection, which has been shown to work well with suspension cell cultures mainly B cells (43), did not give good transient expression. Nor did electroporation which consistently yielded negative results with low viability. Although electroporation is recognized by some as the method of choice for stably transfecting lymphoid cells (6, 25, 27, 44), few reports have defined conditions to perform short term gene transfer in T lymphocytes (24, 25). One problem seems to be in the fragility of the T cells under electroporation conditions. The calcium phosphate coprecipitation, which is widely used for transient as well as permanent gene expression in a variety of cell lines, gave a low level of *cat* expression of pRSVcat in our CTL line. This level is too low if one eventually wants to look at the promoting ability of weak promoters in these cells. The polybrene-assisted gene transfer method which works well with fibroblasts (30) was inefficient in our CTL line.

The DEAE dextran-mediated transfection was at least 100 times that obtained using other methods with the exception of the calcium phosphate coprecipitation where a 10 fold increase was noted. Not only did DEAE dextran give the best transfection efficiency but it also showed best reproducibility as indicated in Table II.1. A standard deviation of less than 1% was noted for experiments performed simultaneously while that for sets performed on different days corresponded to 5-8%. Novak and Rothenberg (10) published a report defining the conditions for short term transfection of MTL 2.8.2 cytotoxic T cells using protoplast fusion. However, their optimized protocol yielded only 9.3% chloramphenicol acetylation using pRSVcat as the expression vector and we believed this would be too low for the weaker promoters to be used in future experiments. We successfully and routinely obtained, during our trial experiments, over 30% acetylation with the same reporter plasmid without optimization of the dextran protocol. Interestingly, the same authors mentioned that in their hands the DEAE dextran-mediated gene transfer did not result in any significant DNA uptake in MTL 2.8.2 cells and concentrations of DEAE dextran between 10 to 100 μg/ml were acutely toxic to the T cells. The differences noted between their results and ours for MTL 2.8.2 cells may be due to the degree of competence of their cells. As indicated below, the maintenance of these cells is an important key to reproducibility and high efficiency of transfection.

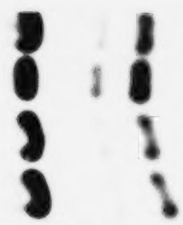
While selecting the proper transfection protocol for MTL 2.8.2 cells, we used both pSV2cat and pRSVcat as control expression vectors. We observed that the level of *cat* expression driven by the Rous sarcoma virus long terminal repeat was much higher than that of pSV2cat, i.e. consistently 10 fold that of SV40 early region. Other investigators reported similar observations with different cell lines (10,34). We therefore decided to use pRSVcat as our reporter expression vector for the optimization of the DEAE dextran-mediated gene transfer.

2. *Optimization of the basic DEAE dextran protocol for MTL 2.8.2*

Many variants of the dextran method have been published but their modifications apply to fibroblasts (32) or myeloma cells (31). In this study, we have utilized the basic DEAE dextran protocol of Fujita *et al.* (20), tried various modifications and have established the parameters deemed critical to the attainment of consistently elevated transfection efficiencies in cytotoxic T cells. Among the various parameters that were examined in order to maximize DNA uptake and *cat* gene expression in MTL 2.8.2 cells: cell density, amount of transfected DNA, concentration of adsorbant agent, adsorption period: length and temperature, permeabilization step: length and temperature, permeabilizing agent: types and concentration, chemical modulators of transcription: types and concentration, length of recovery period were studied. Most transfection protocols involve three phases 1) adsorption, 2) permeabilization, 3) recovery and expression. Many investigators incorporated a DMSO shock (usually between 8-10% for 2 minutes at room temperature) into their basic procedures to enhance DNA uptake (32,45,46). DMSO in many instances appeared to be the permeabilizing agent of choice. For this reason, we decided to test each parameter (except cell density) in the absence and the presence of 8% DMSO as a preliminary attempt to increase *cat* activity.

a. *Cell density*

Non-transformed T cells such as MTL 2.8.2 differ from transformed T cells in that they require constant exposure to cytokines such as IL2 for their growth. The best results in transfection experiments are invariably obtained when the recipient cells are growing exponentially (27,47). To determine the appropriate cell density at which optimal transfection efficiency would be achieved, subconfluent cultures of MTL 2.8.2 maintained in IL2 (30 units/ml) were collected and replated at a density of 4 x 10^4 cells/ml (1 x 10^6 cells/75 cm^2 flask) in the presence of IL2 (30 units/ml). Cells were incubated at 37°C in 5% CO_2 for either 1, 2, 3 or 4 days, transfected using suboptimal conditions for DEAE dextran and then left at 37°C in the 5% CO_2 incubator for 48 hours before being processed for *cat* assays. As shown in Figure II.1, the murine cytotoxic T cell line MTL 2.8.2 appeared most receptive to the DEAE dextran transfection regimen 2 days after its passage in IL2 when cell density reached 1.5 x 10^5 cells/ml. Consequently, cells were routinely plated out at 4 x 10^4 cells/ml and used two days later in all subsequent experiments. Loyter *et al.* (47) suggested that to increase gene transfer frequency, it was necessary to facilitate passage of DNA from the cytoplasm to the nucleus. One possible and easy way to achieve this is to perform the transfection with cells in an exponential growth phase where the percentage of dividing cells is the highest. Cells in mitotic phase are lacking a nuclear membrane and it would appear that it is easier for the transfected DNA to gain access to the nuclear matrix when this extra membrane barrier is absent. Potter *et al.* (27) observed a 3- to 10-fold increase in transfection efficiency using cells halted in metaphase with colcemide. The two fold increase in *cat* activity noted between day 2 and days 1, 3 and 4 is very significant if one considers examining the strength of endogenous cellular promoters



Cell Density (cells / ml)	Days in IL2	CAT Activity (% Acetylation)
4.0×10^4	1	15
1.5×10^5	2	30
4.5×10^5	3	18
8.5×10^5	4	15

FIGURE II.1 Degree of competence of MTL2.8.2 cells in relation to time since last passage in IL2. Cells were seeded at 4×10^4 cells/ml and were left in IL2 (30 units/ml) for the various times indicated before harvest for transfection. Transfection was achieved with 10 µg of pRSVcat and 500 µg/ml of DEAE dextran for 30 minutes at room temperature. Forty eight hours after adsorption, cells were processed for *cat* activity. Results are expressed as the percentage of chloramphenicol converted to its acetylated forms.

such as the CTL-specific serine protease promoters which might be weak compared to the very strong viral promoter RSV constitutively expressed at high levels in a variety of cells.

The DEAE dextran provided reproducible results in any given series of experiments evidenced by the small standard deviations shown. However, this consistency was largely contingent upon the cell density at time of replating prior to gene transfer. The cell density during maintenance of MTL 2.8.2 was a major key to reproducibility. We observed large variations in transfection efficiencies when MTL 2.8.2 cells used for replating prior to transfection were maintained at high densities such as 3.2 to 4 x 10^5 cell/ml (data not shown). The degree of variation ranged from 50-60%. MTL 2.8.2 cultures to be transfected were never allowed to reach confluency and were passaged every second or third day if necessary.

b. *Amount of input DNA*

Many investigators have used between 1.5 and 20 µg of plasmid DNA during transfection assays with dextran (18, 19, 32, 48). Other methods require between 20 ng (polybrene (30)) and up to 120 µg (electroporation (5,49)) of donor DNA. We were interested in determining the minimal and maximal amount of donor DNA that could be used in order to obtain a detectable level of transient *cat* activity. The relationship between the amount of transfected pRSVcat and the resultant level of *cat* expression in MTL 2.8.2 is shown in Figure II.2. The amount of input plasmid DNA varied from 1 to 50 µg and one series was subjected to a DMSO shock (8% at room temperature for 2 minutes, panel B). The level of *cat* activity paralleled the increase in transfected DNA. However, the relationship was not entirely linear since a two fold increase was not noted between each point. The level of *cat* expression progressively increased up to 10 µg/ml then signs of saturation started to appear leading to a less dramatic stimulation of *cat* activity. Interestingly, a plateau was not reached even at 50 µg where a relative increase in chloramphenicol acetylation of 1.3 (-DMSO) to 2 (+DMSO) times that of 25 µg was

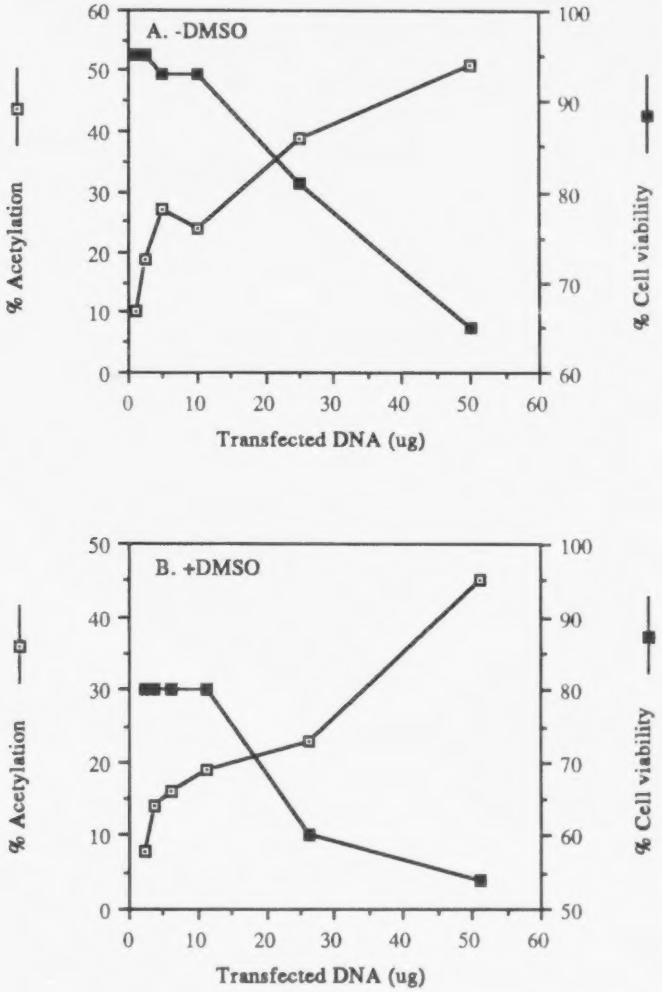


FIGURE II.2 Levels of *cat* activity in MTL2.8.2 cells transfected with increasing amounts of pRSVcat with and without a DMSO shock. Cells were transfected two days after their last passage in IL2 (30 units/ml) with various amounts of DNA and 500 µg/ml DEAE dextran for 30 minutes at room temperature. Immediately after adsorption, cells were shocked with 8% DMSO (v/v) for 2 minutes at room temperature then were washed twice with serum-free medium before plating. Forty eight hours later, cells were collected and whole cell extracts were prepared. *Cat* assays were performed for 4 hours at 37°C using 100-150 µg of protein. Each point represents the average of three or more independent experiments which agreed within 8-10%. Cell viability was determined using the eosin dye exclusion test.

obtained. However, along with this increase in cat activity at 50 μg we also noted a major drop in cell viability. Lieber *et al.* (50) indicated that high concentrations of DNA increase the lytic effect of DEAE dextran by increasing the release of hemoglobin from chicken embryonic erythrocytes leading to excessive lysis. Although erythrocytes are physiologically distinct from cytotoxic T cells, it is reasonable to imagine that a similar situation i.e. the release of some important cellular components could take place in the latter cell type. Recent papers have suggested that the conventional DEAE dextran transfection occurs via transient cell rupture followed by DNA association (50,51). In this instance, it is possible that a large proportion of cells accumulate a high number of plasmid copies and burst while only a certain percentage of cells succeed in taking up a large number of molecules and thus succeed in expressing the reporter gene.

It is a well documented observation that an improvement in *cat* gene expression usually corresponds to a major compromise in cell viability (30,44). In order to keep the transfection experiments on a reasonable scale, we opted to choose as the optimal amount of input DNA, 10-15 μg. This allowed us to recover 80 to 90% of the cells and have enough material to perform various assays on the same transfection samples. *Cat* activity was detected with 1 μg of DNA but the signal was too low for our future purposes. Using 20 μg or more did not offer any advantages considering the reductions in cell viability.

In addition, the enhancement in *cat* activity appeared to be dependent on the *cat* vector concentration rather than on the amount of total DNA introduced in the recipient cells. When pUC19 (unrelated plasmid) was used to increase the amount of DNA from 5 to 10 μg and 10 to 25 μg, the signals obtained corresponded to those of pRSVcat used at 5 or 10 μg (data not shown). The 8% DMSO shock performed at room temperature for 2 minutes did not improve transient *cat* gene expression. Identical signals were detected for both series despite the permeabilization step with DMSO. If anything, the cell viability was lower when the cells were treated with the permeabilization agent.

As expected, very different kinetics were observed with other cell types. Using the osmotic transfection technique, Lieber *et al.* (50) found that the relationship between input DNA and transformation efficiency (*cat* activity or number of colonies) was linear up to 3 µg/ml DNA for chicken embryonic erythrocytes, then there was a decline in expression. For NIH3T3 cells, saturation was reached at about 50 ng/ml form 1 DNA (30). A linear relationship was observed for the mouse lymphoma cell line M12 up to 80 µg/ml DNA using electroporation, then expression declined (25). The plateau of *cat* gene expression for L cells transfected with DEAE dextran was shown to start above 8 µg/ml (46).

At this stage, we wished to determine the copy number of the transfected plasmid DNA in the MTL 2.8.2 cells. This would enable us to determine if the number of cell surface sites for adsorption were saturable at 10 to 15 µg of DNA or if saturation in the level of *cat* activity resulted in the limiting capacity of the transcription/translation machinery of the MTL 2.8.2 cells. To this end, cells were transfected under suboptimal conditions with increasing amounts of pRSVcat supercoiled DNA. Transfectants were collected 48 hours post-transfection, in the presence of 20 mM EDTA, and low molecular weight DNA was purified from Hirt supernatants (41). This DNA was subjected to conventional dot blot analysis using [^{32}P]nick-translated pRSVcat plasmid as the probe. Quantitation of the average copy number per transformed cell was determined by using pRSVcat plasmid DNA as standards. Picogram amounts of pRSVcat equivalent to 10^6 cells having 10 copies/cell or 25, 50, 75, 100, 250, 500, 600 and 700 were used. The same number of cells from the transfected MTL2.8.2 samples were spotted on the Nylon membrane along with the standards. As seen in Figure II.3, MTL 2.8.2 cells transfected with 1, 5, 10 and 20 µg of form 1 DNA contained respectively 20-25, 100-250, 600-700 and above 700 copies of plasmid sequences per cell. These figures indicate internalized DNA molecules as the cell surface bound DNA is dissociated upon EDTA treatment. Figure II.2 showed a decrease in the slope above 10 µg DNA but this set of results indicated that even at 20 µg, the MTL 2.8.2 cells appear to take up plasmid DNA copies

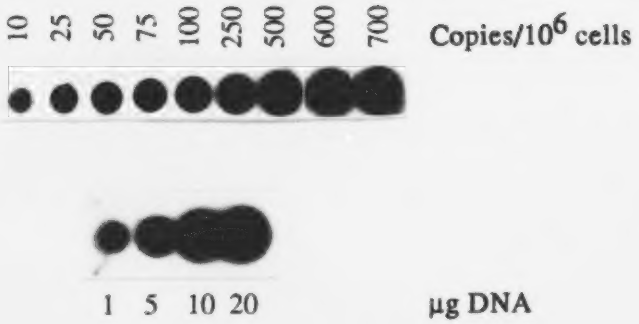


FIGURE II.3 Determination of the number of pRSVcat copies in MTL2.8.2 cells transfected with 500 µg/ml DEAE dextran and increasing amounts of DNA. Transfection was achieved as indicated in legend of Figure II.2. At harvest, cells were washed twice with PBS and low molecular weight DNA was extracted from these cell pellets as detailed by Hirt (41). Standards for the number of copies were prepared using pRSVcat and an equivalent of 1 copy/10^6 cells were set as the base for the calculations. Each dot represents the equivalent of 10^6 cells.

very efficiently using DEAE dextran. Thus the cell surface sites for adsorption of DNA/DEAE dextran complexes are not saturated at 20 µg DNA. The decrease in *cat* gene expression therefore probably resulted from the incapacity of the transcription/translation system to cope with so many DNA copies. We cannot exclude the possibility that although more DNA copies find their way into the cell cytoplasm, the percentage that moves to the nucleus may be low. Furthermore, it is possible that only a certain percentage of this DNA in the nucleus will be in a conformation compatible with transient gene expression. Indeed, Loyter *et al.* (47) have shown that from the 7.5% transfected DNA that gets inside the cells using calcium phosphate precipitates, only 1-5% of input DNA gets to the nucleus. Similar results were obtained by Reeves *et al.* (52). Even microinjecting DNA into the nucleus did not seem to increase the percentage of DNA molecules competent for expression (47). Although it has been difficult to determine the reasons why most of the input DNA never enters the nucleus, a few reports indicated that transfected DNA is subjected to a high mutation frequency shortly after its arrival into the nucleus (53,54). In addition, partial degradation or deletions were found in input DNA isolated from the cytoplasm of transfected cells (53). It is believed that the degradative enzymes and low pH of the lysosomes, vehicles for transfected DNA, damage the DNA in the cytoplasm while the initial lack of a complete chromatin structure renders this DNA even more susceptible to attack in the nucleus (53). It is still unclear where (i.e. in which cell compartments) the damage occurs but transfected DNA molecules quickly undergo changes which compromise their transcription and/or translation (54). Thus, these high copy numbers estimated for MTL2.8.2 cells do not allow us to determine if 100% of the cells took up 250 copies or only 25% of the cells introduced DNA sequences at 1000 copies per cell. And more importantly, they do not allow us to determine transfection efficiency i.e the percentage of DNA molecules that moved to the nucleus and were expressed.

c. *The concentration of DEAE dextran*

Another parameter which undoubtedly affects the level of transient *cat* expression is the concentration of the adsorbant agent. To study this parameter, we utilized concentrations of DEAE dextran in the range of 100 to 2000 μg/ml. MTL 2.8.2 cells were transfected with 10 μg of pRSVcat DNA. The adsorption was set for 30 min. at room temperature in the presence of various concentrations of DEAE dextran and again one series was subjected to the 8% DMSO shock. Figure II.4 indicates that MTL 2.8.2 cells can tolerate up to 2000 μg/ml of DEAE dextran in the transfection cocktail, however, signs of significant cell death were observed at this concentration. The increase in the level of *cat* activity paralleled the increase in the DEAE dextran concentration up to 500 μg/ml then toxicity started to interfere and *cat* gene expression was compromised. Shocking the cells with 8% DMSO following adsorption did not improve the percentage of chloramphenicol acetylation. For the cytotoxic T cell line MTL 2.8.2, DNA uptake was optimal at 500 μg/ml of DEAE dextran and this concentration was used in all subsequent experiments. Clearly, DEAE dextran is less toxic than lipofectin or polybrene.

d. *DNA adsorption: time and temperature*

Sompayrac and Donna (55) reported that exposure to DEAE dextran concentrations above 400 μg/ml for long periods of time such as 8 hours significantly lowered cell survival. They then modified their protocol to circumvent the toxic effects attributed to this chemical facilitator by exposing the cells to lower concentrations of dextran for longer periods of time. Many investigators have chosen to do the same (46,56). We examined the effect of exposing the MTL 2.8.2 cells to the transfection cocktail (DNA + DEAE dextran) for various lengths of time at either 37°C in 5% CO_2 atmosphere or at room temperature in the laminar flow hood. Cells were transfected with 10 μg of standard plasmid DNA using 500 μg/ml of DEAE dextran and were left for various times at room temperature or 37°C before being washed or shocked with 8% DMSO. The results

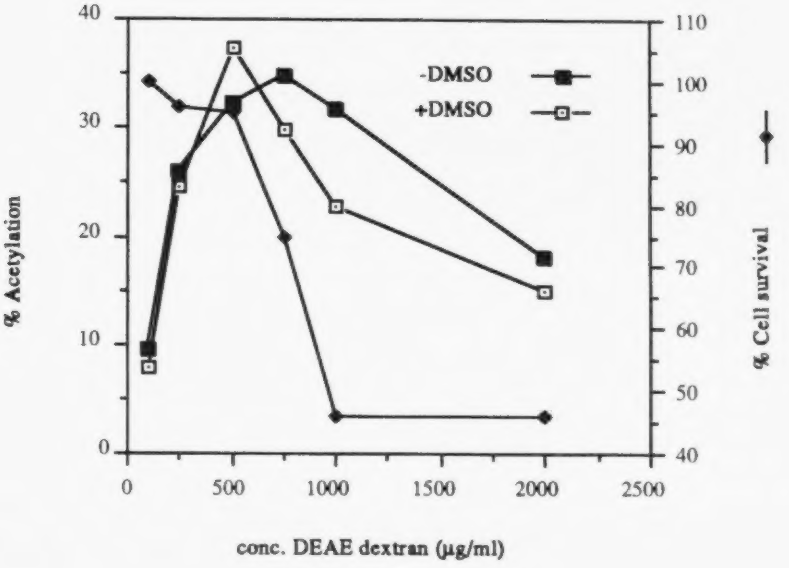


FIGURE II.4 Determination of the optimal concentration of DEAE dextran for transfection into MTL2.8.2 cells. The concentration of DEAE dextran was varied from 100 to 2000 µg/ml and the amount of pRSVcat was kept at 10 µg. Adsorption was set for 30 minutes at room temperature and was immediately followed by a 2 minute DMSO (8%) shock at room temperature for one series of samples. Forty eight hours post-transfection, cells were collected, counted and processed for *cat* activity. Results are the mean of triplicate experiments and standard deviations were between 5-10%.

presented in Table II.2 indicate how quickly expression can be detected. After a 5 minute exposure to the DNA/DEAE dextran cocktail it appeared that 60% of maximal activity is achieved. This number increased to 73% after 10 minutes and 100% at 15 minutes. Within 15 minutes, the level of *cat* activity reached a saturation point, showing no increase even after 4 hours of adsorption. In addition, exposing the cells to 500 µg/ml for 4 hours did not result in any reduction in cell viability.

DNA uptake was not dependent on temperature since the levels of *cat* gene expression obtained following transfections of pRSVcat at 22-25°C or 37°C were identical. These various combinations did not improve the ability of DMSO to enhance transient *cat* expression. Compared to most gene transfer protocols described to date, this adapted dextran procedure requires the shortest adsorption time. Many investigators have used 30 minutes up to 24 hours as adsorption times for DEAE dextran-mediated gene transfer (18, 19, 22, 46, 48, 56). Lipofection, polybrene-assisted transfer and calcium coprecipitation all demand longer adsorption periods to achieve the best transformation efficiencies (28, 29, 30, 37, 43). This short period can be extremely advantageous when transfecting type 1 (antigen- and IL2-dependent) cells which are even more sensitive than type 2 (antigen-independent, IL2-dependent) T cell lines to transfection regimen.

e. *Permeabilization step: time and strength of shock*

Various treatments aimed at improving DNA uptake or *cat* gene expression have been described by others (19, 30, 31, 32, 34, 42, 46, 48). The mechanism of action of these chemical "facilitators" is unclear but their positive modulatory effect on the expression of transfected donor DNA in various recipient cells is well documented (5- to 100-fold enhancement using DMSO (30, 32, 45, 46), 5- to 200-fold enhancement with glycerol (32, 57). We sought to determine if any of these "facilitators" would also be efficient in the cytotoxic T cell line MTL 2.8.2. In the previous series of experiments we used an 8% DMSO shock at room temperature for 2 minutes mainly because numerous reports had

Adsorption time (min.)	Temperature of adsorption process: 22-25°C, Permeabilization treatment: -DMSO	22-25°C +DMSO	37°C -DMSO	37°C +DMSO
5	18	16	ND	ND
10	21	16	ND	ND
15	29	25	31	24
30	33	26	29	26
60	31	28	28	21
240	30	24	29	20

TABLE II.2 Levels of transient *cat* gene expression in MTL2.8.2 cytotoxic T cells following different adsorption regimens. Cells (1.25 x 10^7) were incubated with 10 µg pRSVcat and 500 µg/ml DEAE dextran for the various lengths of time indicated at room temperature (laminar flow hood) or at 37°C (5% CO_2 incubator). Following the adsorption period, one set of samples for each temperature was subjected to a 2 minute DMSO (8%) shock at room temperature. Following washes and further incubation for 48 hours at 37°C/5% CO_2, cells were collected, counted and assayed for *cat* activity. These absolute values represent the mean of four independent experiments. The degree of variation between them was 10%. ND: not determined.

suggested such treatment improved DNA uptake (32, 45, 46). However, our observations so far indicated that this shock did not affect transfection efficiency in MTL 2.8.2. No difference was noted between samples exposed to DMSO and those left unpermeabilized (see Figures II.2, II.4 and Table II.2). To confirm this indifference towards such treatment we decided to expand the range of DMSO used from 5 to 15% and shocked the cells for 2, 5, 10 or 30 minutes at room temperature (laminar flow hood) or at 37°C in 5% CO_2 atmosphere. Results are summarized in Figure II.5. We observed a small increase (1.3 fold) in the level of *cat* activity over that of the unshocked cells (baseline: 30%) when 8%

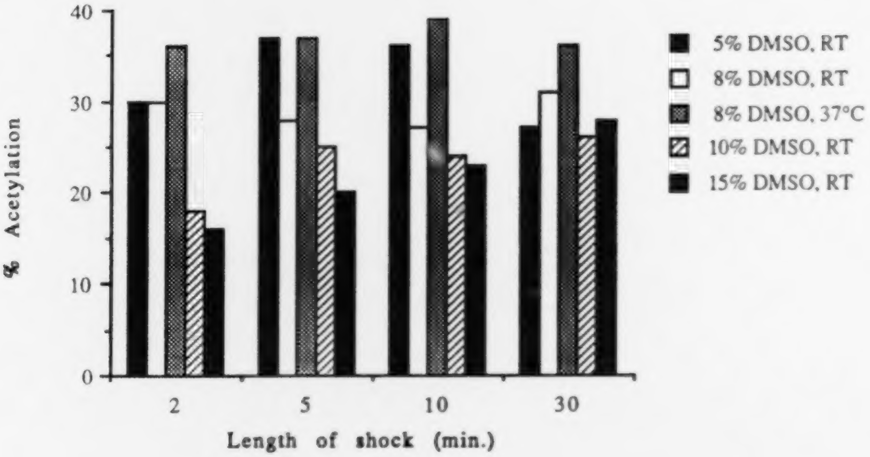


FIGURE II.5 Assay of *cat* activity following transfection of pRSVcat into MTL2.8.2 cells subjected to different DMSO shocks. 10 µg plasmid DNA and 500 µg/ml DEAE dextran were used in this series of experiments. The adsorption period was set for 15 minutes at room temperature. Following this step, different concentrations of DMSO were added directly to the cell/DNA/DEAE dextran cocktail for the indicated lengths of time at room temperature (laminar flow hood) or 37°C (5% CO_2 incubator). Cells were then washed twice and plated for 48 hours before being assayed for *cat* activity; expressed here as the percentage of conversion of chloramphenicol to its monoacetylated forms. Note: the baseline level of *cat* activity for cells not treated with DMSO was 30 ± 3%. Standard deviations were between 10-12%.

DMSO was utilized for either 2, 5, 10 or 30 minutes at 37°C/5% CO_2. This was interesting since the same permeabilization performed at room temperature gave absolutely no increase in the percentage of acetylation. A possible explanation could be that at 37°C the cell membrane fluidity is such that DNA bound to the surface can more easily find its way in the cytoplasm than in the nucleus. Despite this slight improvement in *cat* transient expression, no significant changes in *cat* activity were noted for any of the combinations tried. However, signs of toxicity were evident above 8% DMSO where only 40% of the cells were recovered at harvest (data not shown). MTL 2.8.2 cells appear to be very sensitive to DMSO since many cell types can easily tolerate up to 15% DMSO (30, 58, 59). In addition, the temperature at which the shock was performed did not significantly improve DNA uptake.

We also examined the effect of substituting DMSO with glycerol or polyethylene glycol (PEG) in increasing transfected DNA uptake. These permeabilization agents have been used on other cell types and gave strong stimulatory effects on *cat* expression (31, 32, 34, 42, 57, 60). Cells responded very poorly to glycerol shocks 2.5 to 15% performed at room temperature or at 37°C/5% CO_2 for 2, 5 or 10 minutes. Levels of *cat* activity obtained were consistently lower than that of cells not treated with glycerol (data not shown). Considering the high degree of toxicity exhibited by glycerol even within the lowest range of concentration and the low level of tolerance of MTL 2.8.2 cells for this permeabilization agent, we decided not to pursue this series of transfection experiments any further. The results with PEG were similar to those observed using glycerol.

f. *Other chemical agents to improve transfection efficiency*

Many investigators have reported that sodium butyrate (61, 62) or chloroquine diphosphate (63) significantly increase transient gene expression. It is believed that chloroquine prevents lysosomal degradation of transfected DNA allowing it to reach the nucleus intact for gene expression (63). Recently, Frankel and Pabo (64) observed that tat

(HIV transcriptional activator) was stabilized by chloroquine. It is therefore possible that this compound could also protect other key transcriptional factors from proteolytic degradation. The detailed mechanism of action of sodium butyrate remains unclear. However, many reports have provided evidence that the enhancement seen with butyrate is mediated through effects on cellular chromatin by inhibition of histone deacetylase(s), resulting in the accumulation of hyperacetylated core-particle histones (52, 65, 66, 67). Very recently, work done by Bresnick *et al.* (67) strongly suggested that changes in the acetylation status of the chromatin can have an important impact on allowing transcription factors to find their way to their specific binding sequences. Because of the obvious improvement in gene expression in cells treated with these agents, we decided to test if MTL2.8.2 cells would respond positively to such regimen.

MTL 2.8.2 cells were transfected and treated as indicated in Materials and Methods and one series of samples was permeabilized with 8% DMSO for 2 minutes at 37°C before chloroquine treatment. As shown in Table II.3, the presence of chloroquine diphosphate had no effect on *cat* gene expression for any combinations examined. Signs of toxicity were observed at concentrations higher than 50 µM at which only 40% of the cells were recovered. Pretreating the cells with DMSO did not improve *cat* activity (data not shown).

Interestingly, although the cytotoxic T cells did not respond to chloroquine diphosphate, the treatment with sodium butyrate significantly improve their ability to transcribe the *cat* gene (Figure II.6). Increasing the concentration of butyrate from 1 to 10 mM resulted in a five fold stimulation after 12 hours treatment (12% at 1 mM and 58% at 10 mM). The degree of toxicity increased proportionally: 68% of cells were recovered when 1 mM butyrate was used and only 42% at the 10 mM concentration. This stimulatory effect was not observed when cells were subjected to butyrate for only 4 hours.

The cells were also shocked with 8% DMSO at 37°C prior to exposure to butyrate to determine if transcription could be enhanced. As indicated in Figure II.6, the increase in

Concentration chloroquine diphosphate (μM)	Length of treatment (hrs)	*Cat* activity chloramphenicol acetylation (%) Permeabilization treatment -DMSO	*Cat* activity chloramphenicol acetylation (%) Permeabilization treatment +DMSO
0		22	22
25	2	32	23
25	4	27	30
50	2	21	19
50	4	27	25
75	2	19	15
75	4	16	29
100	1	20	26
100	2	19	25
100	3	15	19
100	4	16	12

TABLE II.3 Effects of chloroquine diphosphate on levels of *cat* activity in MTL2.8.2 cells. MTL 2.8.2 cells were transfected with 10 μg pRSVcat and 500 μg/ml DEAE dextran for 15 minutes at room temperature. After washes, they were plated in complete medium supplemented with various concentrations of chloroquine diphosphate as indicated and left at 37°C for the lengths of time shown. Cells were then washed and incubated in complete medium for 48 hours before *cat* determination. One set of samples was pretreated with DMSO (8%) at 37°C (see Materials and Methods) before chloroquine treatment.

the level of *cat* gene expression remained constant or was down 1.6 fold that observed previously without the DMSO for the various concentrations of butyrate used. This dual treatment appeared very toxic to the cells since only 50% were recovered at 1 mM, 30% at 4 mM and 10% at 10 mM concentration.

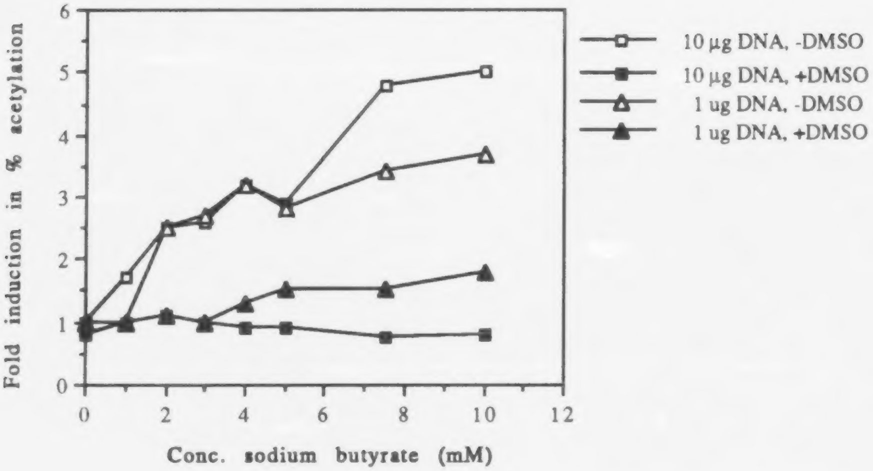


FIGURE II.6 Effects of sodium butyrate on transient *cat* gene expression in MTL2.8.2 cells. Cells were transfected with 10 µg pRSVcat and 500 µg/ml DEAE dextran for 15 minutes at room temperature. Following washes, cells were resuspended in complete medium (RHFM+ 30 units/ml of IL2) and dispensed into plates containing medium supplemented with various concentrations of sodium butyrate as indicated. After 12 hours at 37°C/5% CO_2 the medium was removed and cells were fed fresh medium for a further 36 hours, at which time extracts were prepared for assaying *cat* activity. Cell viability was determined after removal of the sodium butyrate and also 36 hours later at harvest. The effect of sodium butyrate on the level of *cat* activity is expressed as the fold induction over that of the untreated cells.

Reeves *et al.* (52) indicated that the effect of sodium butyrate decreased with increasing concentrations of input DNA (16 fold with 1 µg/ml transfected DNA versus 1 fold with 10 µg/ml) in CV-1 cells transfected with the calcium phosphate coprecipitation technique. Although we were following a different gene transfer protocol, we looked at the effect of adding only 1 µg of pRSVcat instead of 10 µg in the transfection assays. In this instance we observed a stimulation up to four fold (at 10 mM, 12% to 44%) representing a smaller improvement than that obtained using 10 µg of input DNA. Again the pretreatment with 8% DMSO at 37°C had no enhancement or had a negative effect on *cat* gene expression. The difference noted in the percentage of chloramphenicol acetylation when going from 1 to 10 µg of transfected DNA is very small and in both instances, *cat* activity increased proportionally to the concentrations of butyrate. Our data disagree with Reeves *et al.* (52) observations although this discrepancy may have arisen from the different recipient cell lines and/or the different methods of transfection used in the studies.

Recent reports have shown that sodium butyrate increases the stability of mRNA (68) and in some instances increases the transcriptional rate of certain genes (69). These effects resulted in increases in steady-state levels of mRNAs for a number of genes (70, 71) or in increases in the protein levels (72, 73). Future experiments are required to assess which of these situations is responsible for the 5 fold enhancement seen in our transient *cat* gene expression.

g. *Length of cell recovery after transfection*

Every cell line responds differently to a transfection regimen. Each requires a different period of recovery after transfection before transcription starts; adsorbed DNA has to pass through the cytoplasm and then be transported into the nucleus in order to be transcribed (47). Previous observations indicated that 24 hours were required to allow transfected DNA to be transcribed and translated. Figure II.7 shows the dependence of the level of *cat* expression on the time interval between the actual transfection (DEAE dextran

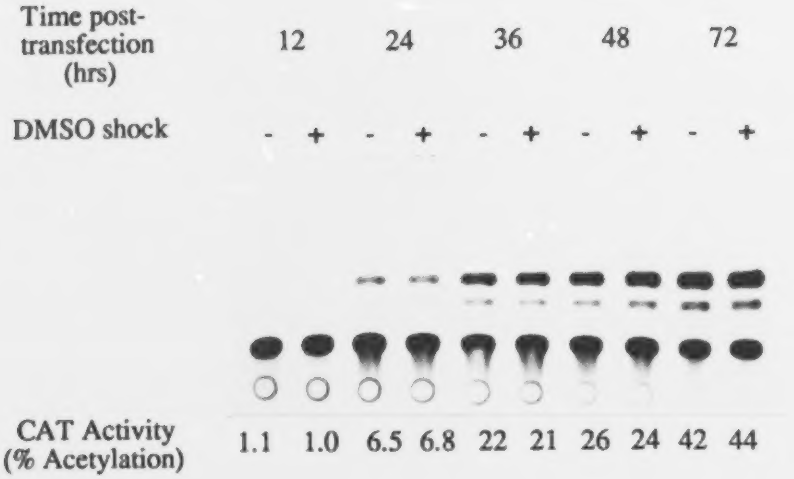

Time post-transfection (hrs)
12
24
36
48
72
DMSO shock
- + - + - + - + - +
CAT Activity (% Acetylation)
1.1 1.0 6.5 6.8 22 21 26 24 42 44

FIGURE II.7 Determination of the optimal peak of transient *cat* expression driven by RSV in MTL2.8.2 cells. Cells were transfected following optimal conditions i.e. 10 µg pRSVcat, 500 µg/ml DEAE dextran for 15 minutes at room temperature. Cells were collected at times indicated after transfection and *cat* activity was assayed.

treatment) and cell harvest. *Cat* gene expression increased with time, increasing from 1 to 7% within 24 hours after transfection, from 7 to 22% 12 hours later then to 29% at 48 hours post-transfection and finally reached a maximum of 44% at 72 hours. At this point the high cell density gave rise to cell death. Similar observations have been reported with different cell lines (30, 46). Thus we decided to use 48 hours as the read out time in future experiments.

3. *Comparison of the strength of the cytotoxic cell serine protease 5'-flanking sequences with other viral and cellular regulatory regions*

In optimizing the DEAE dextran transfection protocol for the cytotoxic T cell line MTL2.8.2, our intentions were to design a system that would allow us to study in detail the CTL-specific serine protease promoters and their regulatory sequences. Our first analysis focused on two of the CTL-serine protease genes isolated and characterized in our laboratory and referred to as B10 and C11 (75). We examined the strength of B10 and C11 promoters by placing the 5'-flanking region of both genes immediately upstream of the promoterless vector pGEM2cat (see Materials and Methods). These constructs were introduced into MTL2.8.2 cells following the optimized DEAE dextran strategy outlined earlier. Other *cat* expression vectors were also transfected into the same recipient cell line. In order to determine if other *cat*-bearing plasmids are positively affected by sodium butyrate like pRSVcat, we performed the same series of transfection assays in the absence or the presence of 7.5 mM butyrate for 12 hours. Table II.4 summarizes the results of this analysis. First, both B10 and C11 promoters are weaker than the two strong viral promoters. The thymidine kinase promoter is also quite powerful and both serine protease 5'-flanking regions exhibited lower activity. B10 and C11 are as good as the basal c-fos in stimulating transcription from the *cat* gene. One important aspect to keep in mind when comparing strength of various 5'-flanking regions is that this strength is dependent on the recipient cells in which these regions are tested. Some flanking

	Cat activity chloramphenicol acetylation (%)		
Vectors	-butyrate	+butyrate	Total induction
pB10 1080cat	1.2	1.6	1.3
pC11 896cat	1.0	5.7	5.7
pRSVcat	12	58	4.8
pSV2cat	5.4	32	5.9
pA10cat2	2.5	2.1	0.8
pTKcat	5.3	7.4	1.4
pFCΔ56	0.9	1.1	1.2

TABLE II.4 Comparison of the strengths of B10 and C11 serine protease 5'-flanking sequences with those of other viral and cellular regulatory regions. MTL2.8.2 cells were transfected with 10 μg each of a variety of plasmids in the presence of 500 μg/ml DEAE dextran for 15 minutes at room temperature. Following adsorption and washes, cells were plated in medium containing 0 mM or 7.5 mM sodium butyrate and incubated for 12 hours at 37°C in a 5% CO_2 incubator. Following media change cells were incubated for a further 36 hours before harvest. Values shown represent absolute values for *cat* activity.

sequences may appear very powerful in a variety of cell lines because their requirements for transcriptional factors are minimal i.e. their DNA-binding proteins are ubiquitous and found in abundancy in all cells. On the other hand, some 5'-flanking regions may tend to show lower strength because their transcriptional factors are quite distinct and in low abundance and may be found only in certain cell lines.

One very interesting observation was made when the cells were treated with butyrate for 12 hours during the transfection process. We noted a large difference between B10 and C11 relative strengths. While the B10 5'-flanking region was capable of stimulating transcription of the *cat* gene as efficiently as the c-fos promoter, the C11 promoter region did it five times more efficiently. Table II.4 indicates that, following exposure to butyrate for 12 hours at 37°C, the C11 5'-flank promotes transcription better than tk and SV40 early region lacking the enhancers but is still weaker than RSV and SV40 entire regulatory regions. On the other hand, B10 is weaker than all viral and cellular regulatory sequences tested except for the SV40 early region depleted of enhancers. We have also observed that although RSV, SV40 entire regulatory regions were responsive to butyrate and showed an increase in their ability to drive transient *cat* expression, it did not appear to be the case for tk, the basal c-fos and SV40 5'-flanking region lacking enhancers. This difference could reflect the different requirements in transcriptional factors for each of these promoters and flanking sequences.

This differential enhancement in *cat* activity for the B10 and C11 5'-flanking sequences with sodium butyrate was surprising and very interesting. Sodium butyrate is believed to modify the chromatin structure but an apparently limited number of genes are butyrate responsive (61, 62, 67, 69). Our results suggest that B10 and C11 promoter sequences differ structurally or some of the transcriptional factors that bind to them are distinct. This distinction is presently under investigation.

In summary, we have adapted the DEAE dextran-mediated gene transfer protocol to successfully obtain high and reproducible levels of transient *cat* gene expression in a variety of murine cytotoxic T cells. We have shown that the cells were most receptive to the transfection regimen two days after their latest passage in IL2, presumably the time at which the number of mitotic figures are the highest. We also demonstrated that optimal *cat* gene expression was obtained using 10-15 μg DNA with 500 μg/ml of DEAE dextran for 15 minutes at room temperature. Adsorption need not be longer and represents to our knowledge the shortest time employed to transfect cells with the exception of electroporation. The transfection efficiency in CTLs was not improved after treatment of the cells with either DMSO, glycerol, PEG or chloroquine diphosphate. However, sodium butyrate at a concentration of 7.5 mM exposed to the cells for 12 hours at 37°C increased the level of *cat* activity by a factor of five. This protocol allowed us to achieve significant level of *cat* gene expression using CTL-specific cellular promoters and provides us with an excellent means to start delineating the differences between two of our CTL-specific serine protease promoters B10 and C11. Although the protocol was not rigorously optimized for other T cell lines we have shown that it also works well with three independent antigen-dependent and IL2-dependent T cell clones (data not shown). In addition, this method will provide an experimental system for the identification of the regulatory sequences that control the expression of any other CTL-specific genes. It may also be particularly useful for manipulating the phenotype of this most interesting cell type.

BIBLIOGRAPHY

1. Kaye, J. and Hedrick, S.M. (1988). Analysis of specificity for antigen, Mls, and allogeneic MHC by transfer of T-cell receptor α- and β-chain genes. *Nature* **336**:580-583.

2. Nalefski, E.A., Wong, J.G.P. and Rao, A. (1990). Amino acid substitutions in the first complementarity-determining region of a murine T-cell receptor α chain affect antigen-major histocompatibility complex recognition. *J. Biol. Chem.* **265**:8842-8846.

3. Saga, Y., Lee, J.S., Saraiya, C. and Boyse, E.A. (1990). Regulation of alternative splicing in the generation of isoforms of the mouse Ly-5 (CD45) glycoprotein. *Proc. Natl. Acad. Sci. USA* **87**:3728-3732.

4. Goverman, J., Gomez, S.M., Segesman, K.D., Hunkapiller, T., Laug, W.E. and Hood, L. (1990). Chimeric immunoglobulin-T cell receptor proteins form functional receptors: Implications for T cell receptor complex formation and activation. *Cell* **60**:929-939.

5. Doi, T., Hatakeyama, M., Itoh, S. and Taniguchi, T. (1989). Transient induction of IL2 receptor in cultured T cell lines by HTLV-1 LTR-linked tax-1 gene. *EMBO J.* **8**:1953-1958.

6. Ohtani, K., Nakamura, M., Saito, S., Nagata, K., Sugamura, K. and Hinuma, Y. (1989). Electroporation: application to human lymphoid cell lines for stable introduction of a transactivator gene of human T-cell leukemia virus type I. *Nucleic Acids Res.* **17**:1589-1604.

7. Reed, J.C., Cuddy, M., Haldar, S., Croce, C., Nowell, P., Makover, D. and Bradley, K. (1990). BCL2-mediated tumorigenicity of a human T-lymphoid cell line: synergy with MYC and inhibition by BCL2 antisense. *Proc. Natl. Acad. Sci. USA* **87**:3660-3664.

8. McCubrey, J., McKearn, J.P. and Köhler, G. (1985). Transformation of B and non-B cell lines with the 2,4,6-trinitrophenyl (TNP)-specific immunoglobulin genes. *Eur. J. Immunol.* **15**:1117-1124.

9. Ochi, A., Hawley, R.G., Shulman, M.J. and Hozumi, N. (1983). Transfer of a cloned immunoglobulin light-chain gene to mutant hybridoma cells restores specific antibody production. *Nature* **302**:340-342.

10. Novak, T.J. and Rothenberg, E.V. (1986). Differential transient and long-term expression of DNA sequences introduced into T-lymphocyte lines. *DNA* **5**:439-451.

11. Hambor, J.E., Hauer, C.A., Shu, H.-K., Groger, R.K., Kaplan, D.R. and Tykocinski, M.L. (1988). Use of an Epstein-Barr virus episomal replicon for antisense RNA-mediated gene inhibition in a human cytotoxic T-cell clone. *Proc. Natl. Acad. Sci. USA* **85**:4010-4014.

12. Berman, J.W., Basch, R.S. and Pellicer, A. (1984). Gene transfer in lymphoid cells: expression of the Thy-1.2 antigen by Thy-1.1 BW5147 lymphoma cells transfected with unfractionated cellular DNA. *Proc. Natl. Acad. Sci. USA* **81**:7176-7179.

13. Hui, K.M., Sim, Tse'F., Foo, T.T. and Oei, A.-A. (1989). Tumor rejection mediated by transfection with allogeneic class I histocompatibility gene. *J. Immunol.* **143**:3835-3843.

14. Kondo, S., Shimizu, A., Maeda, M., Tagaya, Y., Yodoi, J. and Honjo, T. (1986). Expression of functional human interleukin-2 receptor in mouse T cells by cDNA transfection. *Nature* **320**:75-77.

15. Abken, H., Bützler, C. and Willecke, K. (1988). Immortalization of human lymphocytes by transfection with DNA from mouse L929 cytoplasts. *Proc. Natl. Acad. Sci. USA* **85**:468-472.

16. Kasid, A., Morecki, S., Aebersold, P., Cornetta, K., Culver, K., Freeman, S., Director, E., Lotze, M.T., Blaese, R.M., Anderson, W.F. and Rosenberg, S.A. (1990). Human gene transfer: characterization of human tumor-infiltrating lymphocytes as vehicles for retroviral-mediated gene transfer in man. *Proc. Natl. Acad. Sci. USA* **87**:473-477.

17. Miyatake, S., Nishihara, K., Kikuchi, H., Yamashita, J., Namba, Y., Hanaoka, M. and Watanabe, Y. (1990). Efficient tumor suppression by glioma-specific murine cytotoxic T lymphocytes transfected with interferon-γ gene. *J. Natl. Cancer Inst.* **82**:217-220.

18. Miyatake, S., Seiki, M., Yoshida, M. and Arai, K.-I. (1988). T-cell activation signals and human T-cell leukemia virus type I-encoded $p40^x$ protein activate the mouse granulocyte-macrophage colony-stimulating factor gene through a common DNA element. *Mol. Cell. Biol.* **8**:5581-5587.

19. Diamond, D.J., Nelson, F.B. and Reinherz, E.L. (1989). Lineage-specific expression of a T cell receptor variable gene promoter controlled by upstream sequences. *J. Exp. Med.* **169**:1213-1231.

20. Fujita, T., Shibuya, H., Ohashi, T., Yamanishi, K. and Taniguchi, T. (1986). Regulation of human interleukin-2 gene: functional DNA sequences in the 5' flanking region for the gene expression in activated T lymphocytes. *Cell* **46**:401-407.

21. Ratanavongsiri, J., Igarashi, S., Mangal, S., Kilgannon, P., Fu, A. and Fotedar, A. (1990). Transcription of the T cell receptor β-chain gene is controlled by multiple regulatory elements. *J. Immunol.* **144**:1111-1119.

22. Sherman, P.A., Basta, P.V. and Ting, J.P.-Y. (1987). Upstream DNA sequences required for tissue-specific expression of the HLA-DRα gene. *Proc. Natl. Acad. Sci. USA* **84**:4254-4258.

23. Folks, T.M., Clouse, K.A., Justement, J., Rabson, A., Duh, E., Kehrl, J.H. and Fauci, A.S. (1989). Tumor necrosis factor α induces expression of human immunodeficiency virus in a chronically infected T-cell clone. *Proc. Natl. Acad. Sci. USA* **86**:2365-2368.

24. Lowenthal, J.W., Ballard, D.W., Böhnlein, E. and Greene, W.C. (1989). Tumor necrosis factor α induces proteins that bind specifically to κB-like enhancer elements and regulate interleukin 2 receptor α-chain gene expression in primary human T lymphocytes. *Proc. Natl. Acad. Sci. USA* **86**:2331-2335.

25. Toneguzzo, F., Hayday, A.C. and Keating, A. (1986). Electric field-mediated DNA transfer: transient and stable gene expression in human and mouse lymphoid cells. *Mol. Cell. Biol.* **6**:703-706.

26. Luria, S., Gross, G., Horowitz, M. and Givol, D. (1987). Promoter and enhancer elements in the rearranged α chain gene of the human T cell receptor. *EMBO J.* **6**:3307-3312.

27. Potter, H., Weir, L. and Leder, P. (1984). Enhancer-dependent expression of human kappa immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. *Proc. Natl. Acad. Sci. USA* **81**:7161-7165.

28. Felgner, P.L., Gadex,T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M. and Danielsen, M. (1987). Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA* **84**:7413-7417.

29. Graham, F. and van der Eb, A. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**:456-467.

30. Aubin, R.J., Weinfeld, M. and Paterson, M.C. (1988). Factors influencing efficiency and reproducibility of polybrene-assisted gene transfer. *Somatic Cell Molec. Genet.* **14**:155-167.

31. Gopal, T.V. (1985). Gene transfer method for transient gene expression, stable transformation, and cotransformation of suspension cell cultures. *Mol. Cell. Biol.* **5**:1188-1190.

32. Sussman, D.J. and Milman, G. (1984). Short-term, high-efficiency expression of transfected DNA. *Mol. Cell. Biol.* **4**:1641-1648.

33. Bleackley, R. C., Havele, C. and Paetkau, V. (1982). Cellular and molecular properties of an antigen-specific cytotoxic T lymphocyte line. *J. Immunol.* **128**:758-767.

34. Gorman, C.M., Merlino, G.T., Willingham, M.C., Pastan, I. and Howard, B.H. (1982). The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. *Proc. Natl. Acad. Sci. USA* **79**:6777-6781.

35. Gorman, C., Moffat, L. and Howard, B. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* **2**:1044-1051.

36. Chung, J., Sinn, E., Reed, R.R. and Leder, P. (1986). Trans-acting elements modulate expression of the human c-myc gene in Burkitt lymphoma cells. *Proc. Natl. Acad. Sci. USA* **83**:7918-7922.

37. Gilman, M.Z., Wilson, R.N. and Weinberg, R.A. (1986). Multiple protein-binding sites in the 5'-flanking region regulate c-fos expression. *Mol. Cell. Biol.* **6**:4305-4316.

38. Lobe, C.G., Shaw, J., Frégeau, C., Duggan, B., Meier, M., Brewer, A., Upton, C., McFadden, G., Patient, R.K., Paetkau, V. and Bleackley, R.C. (1989). Transcriptional regulation of two cytotoxic T lymphocyte-specific serine protease genes. *Nucleic Acids Res.* **17**:5765-5779.

39. Davis, L.G., Dibner, M.D. and Battey, J.F. (1986). in *Basic Methods in Molecular Biology*, (ed.) Davis, L. G. (Elsevier Science Publishing Co, Inc. New York).

40. Birnboim, H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**:1513-1523.

41. Hirt, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**:365-369.

42. Wallich, R., Bulbuc, N., Hämmerling, G.J., Katzau, S., Segal, S. and Feldman, M. (1985). Abrogation of metastatic properties of tumor cells by *de novo* expression of H-2K antigens following H-2 gene transfection. *Nature* **315**:301-305.

43. Dang, L.H., Michalek, M.T., Takei, F., Benaceraff, B. and Rock, K.L. (1990). Role of ICAM-1 in antigen presentation demonstrated by ICAM-1 defective mutants. *J. Immunol.* **144**:4082-4091.

44. Andreason, G.L. and Evans, G.A. (1989). Optimization of electroporation for transfection of mammalian cell lines. *Anal. Biochem.* **180**:269-275.

45. Stow, N.D. and Wilkie, N.M. (1976). An improved technique for obtaining enhanced infectivity with herpes simplex virus type 1 DNA. *J. Gen. Virol.* **33**:447-458.

46. Lopata, M.A., Cleveland, D.W. and Sollner-Webb, B. (1984). High level transient expression of a chloramphenicol acetyltransferase gene by DEAE dextran-mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. *Nucleic Acids Res.* **12**:5707-5717.

47. Loyter, A., Scangos, G.A. and Ruddle, F.H. (1982). Mechanisms of DNA uptake by mammalian cells: fate of exogenously added DNA monitored by the use of fluorescent dyes. *Proc. Natl. Acad. Sci. USA* **79**:422-426.

48. Seed, B. and Aruffo, A. (1987). Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. *Proc. Natl. Acad. Sci. USA* **84**:3365-3369.

49. Solomon, K.R., Krangel, M.S., McLean, J., Brenner, M.B. and Band, H. (1990). Human T cell receptor-γ and -δ chain pairing analysed by transfection of a T cell receptor-δ negative mutant cell line. *J. Immunol.* **144**:1120-1126.

50. Lieber, M.R., Hesse, J.E., Nickol, J.M. and Felsenfeld, G. (1987). The mechanism of osmotic transfection of avian embryonic erythrocytes: analysis of a system for studying developmental gene expression. *J. Cell Biol.* **105**:1055-1065.

51. Lieber, M.R., Hesse, J.E., Mizuuchi, K. and Gellert, M. (1987). Developmental stage specificity of the lymphoid V(D)J recombination activity. *Genes and Development* **1**:751-761.

52. Reeves, R., Gorman, C.M. and Howard, B. (1985). Minichromosome assembly of non-integrated plasmid DNA transfected into mammalian cells. *Nucleic Acids Res.* **13:**3599-3615.

53. Miller, J.H., Lebkowski, J.S., Greisen, K.S. and Calos, M.P. (1984). Specificity of mutations induced in transfected DNA by mammalian cells. *EMBO J.* **3**:3117-3121.

54. Wake, C.T., Gudewicz, T., Porter, T., White, A. and Wilson, J.H. (1984). How damaged is the biologically active subpopulation of transfected DNA?. *Mol.Cell.Biol.* **4:**387-398.

55. Sompayrac, L.M. and Danna, K.J. (1981). Efficient infection of monkey cells with DNA of simian virus 40. *Proc. Natl. Acad. Sci. USA* **78**:7575-7578.

56. Cereghini, S. and Yaniv, M. (1984). Assembly of transfected DNA into chromatin: structural changes in the origin-promoter-enhancer region upon replication. *EMBO J.* **3**:1243-1253.

57. Fraley, R., Subramani, S., Berg, P. and Papahadjopoulos, D. (1980). Introduction of liposome-encapsulated SV40 DNA into cells. *J. Biol. Chem.* **255**:10431-10435.

58. Randak, C., Brabletz, T., Hergenröther, M., Sobotta, I. and Serfling, E. (1990). Cyclosporin A suppresses the expression of the interleukin-2 gene by inhibiting the binding of lymphocyte-specific factors to the IL-2 enhancer. *EMBO J.* **9**:2529-2536.

59. DiMaio, D., Treisman, R. and Maniatis, T. (1982). Bovine papilloma virus vector that propagates as a plasmid in both mouse and bacterial cells. *Proc. Natl. Acad. Sci. USA* **79**:4030-4034.

60. Parker, B.A. and Stark, G.R. (1979). Regulation of simian virus 40 transcription: sensitive analysis of the RNA species present early in infections by virus or viral DNA. *J. Virol.* **31**:360-368.

61. Gorman, C.M. and Howard, B.H. (1983). Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. *Nucleic Acids Res.* **11**:7631-7648.

62. Bohan, C.A., Robinson, R.A., Luciw, P.A. and Srinivasan, A. (1989). Mutational analysis of sodium butyrate inducible elements in the human immunodeficiency virus type 1 long terminal repeat. *Virology* **172**:573-583.

63. Luthman, H. and Magnusson, G. (1983). High efficiency polyoma DNA transfection of chloroquine treated cells. *Nucleic Acids Res.* **11**.1295-1308.

64. Frankel, A.D. and Pabo, C.O. (1988). Cellular uptake of the *tat* protein from human immunodeficiency virus. *Cell* **55**:1189-1193.

65. Oliva, R., Bazett-Jones, D.P., Locklear, L. and Dixon, G.H. (1990). Histone hyperacetylation can induce unfolding of the nucleosome core particle. *Nucleic Acids Res.* **18**:2739-2747.

66. Partington, G.A., Yarwood, N.J. and Rutherford, T.R. (1984). Human globin gene transcription in injected Xenopus oocytes: enhancement by sodium butyrate. *EMBO J.* **3:**2787-2792.

67. Bresnick, E.H., John, S., Berard, D.S., LeFebvre, P. and Hager, G.L. (1990). Glucocorticoid receptor-dependent disruption of a specific nucleosome on the mouse mammary tumor virus promoter is prevented by sodium butyrate. *Proc. Natl. Acad. Sci. USA* **87**:3977-3981.

68. Tang, S.-J., Ko, L.-W., Wu Lee, Y.-H. and Wang, F.-F. (1990). Induction of *fos* and *sis* proto-oncogenes and genes of the extracellular matrix proteins during butyrate induced glioma differentiation. *Biochim. Biophys. Acta* **1048**:59-65.

69. Birren, B.W. and Herschman, H.R. (1986). Regulation of the rat metallothionein-I gene by sodium butyrate. *Nucleic Acids Res.* **14**:853-867.

70. Yang Chou, J., Sartwell, A.D., Lei, K.-J. and Plouzek, C.A. (1990). Effects of sodium butyrate on the synthesis of human pregnancy-specific β_1-glycoprotein. *J. Biol. Chem.* **265**:8788-8794.

71. Mickley, L.A., Bates, S.E., Richert, N.D., Currier, S., Tanaka, S., Foss, F., Rosen, N. and Fojo, A.T. (1989). Modulation of the expression of a multidrug resistance gene (mdr-1/P-glycoprotein) by differentiating agents. *J. Biol. Chem.* **264**:18031-18040.

72. Perrine, S.P., Miller, B.A., Faller, D.V., Cohen, R.A., Vichinsky, E.P., Hurst, D., Lubin, B.H. and Papayannopoulou, T. (1989). Sodium butyrate enhances fetal globin gene expression in erythroid progenitors of patients with Hb SS and β thalassemia. *Blood* **74**:454-459.

73. Schmidt, R., Cathelineau, C., Cavey, M.T., Dionisius, V., Michel, S., Shroot, B. and Reichert, U. (1989). Sodium butyrate selectively antagonizes the inhibitory effect of retinoids on cornified envelope formation in cultured human keratinocytes. *J. Cell. Physiol.* **140**:281-287.

74. Kopchick, J.J. and Stacey, D.W. (1984). Differences in intracellular DNA ligation after microinjection and transfection. Mol. Cell. Biol. **4**:240-246.

75. Lobe, C.G., Finlay, B.B., Paranchych, W., Paetkau, V.H. and Bleackley, R.C. (1986). Novel serine proteases encoded by two cytotoxic T lymphocyte-specific genes. *Science* **232**:858-861.

APPENDIX TO CHAPTER II

A. INTRODUCTION

Chapter II describes a gene transfer strategy developed for transient expression in cytotoxic T cells. The cytotoxic T cell line that was used in the study was MTL2.8.2. It is a type II cell line and requires only IL2 for its proliferation. Type I cells, on the other hand, depend on both IL2 and antigen for their cell growth. Thus, these cell lines resemble primary cell cultures and represent a more relevant biological system to study gene activation following T cell stimulation. However, the difficulties in obtaining large numbers of cells for experimentation and in achieving good transfection efficiency with these cell lines have essentially prohibited their use. In this section, three independent type I T cell clones were tested for their transfectability with the optimized protocol. We demonstrate that type I cells in their quiescent state show little expression of the RSV driven-*cat* gene but that this level is enhanced following stimulation with IL2 and antigen or IL2 and PMA/dibutyryl cAMP.

B. RESULTS AND DISCUSSION

1. *Quiescent type I T cell clones are amenable to transfection by the adapted DEAE dextran protocol*

Three independent type I cytotoxic T cell clones, namely 129.9(I), 2(I) and 21.9(I) or 85 were produced from CBA/Balb/c mice as described earlier (Havele et al., 1986). They were stimulated with antigen (irradiated Balb/c spleen cells) every 7 days in the presence of 20 units/ml of recombinant human IL2. These cells were forced into quiescence by reducing the concentration of IL2 present in the medium from 20 to 10-12 units/ml for one week. For each clone, 10^7 cells were transfected using the DEAE dextran strategy described in Chapter II. Basically, 15 µg of plasmid pRSVcat was used with 500 µg DEAE dextran in 1 ml of transfection buffer. The adsorption time with the

DNA/dextran cocktail was set for 5 minutes at room temperature instead of 15 (see below) following which the cells were washed twice with serum-free medium. They were then incubated for 48 hours at 37°C in a 5% CO_2 atmosphere in complete medium before *cat* determination. We have previously shown that a 15 minute adsorption period with the DNA/DEAE dextran cocktail was sufficient to reach the saturation level of *cat* activity (see Chapter II). We also demonstrated that the percentage of chloramphenicol acetylation was reasonably high after only 5 minutes in the presence of the transfection cocktail. Because of the low tolerance of the type I cells to transfection buffer and extrenuous manipulations, we decided to minimize cell damage and transfect for 5 minutes. As shown in Figure IIA.1, *cat* activity was detectable in all three clones following this modified method. Previous reports have indicated that no *cat* activity can be detected in primary T cells or T cell clones transfected with other regimen unless these cells are stimulated with IL2 and antigen (Fujita et al., 1986, Novak et al., 1990). The signals obtained in the present *cat* assay suggest that the optimized DEAE dextran protocol for type II cells work well with type I T cell clones.

2. *Transfection of type I cells stimulated with IL2 and antigen*

For this experiment, the type I T cell clone 85 was used. These cells were maintained in RHFM supplemented with 20 units/ml. They were passaged at 5×10^5 cells/ml in low IL2 medium (2 units/ml) for 24 hours to allow them to reach quiescence. Transfections were performed on 1.2×10^7 quiescent cells following the protocol outlined above.

Stimulation of clone 85 was achieved as follows: quiescent cells were seeded in medium containing 20 units/ml of IL2 and irradiated Balb/c spleen cells for two days as described in Havele et al., (1986). The mixed cell population was then fractionated by centrifugation on a Ficoll gradient to remove red blood cells, residual antigen and dead cells. The leukocyte fraction (containing T lymphocytes) was then transferred to complete

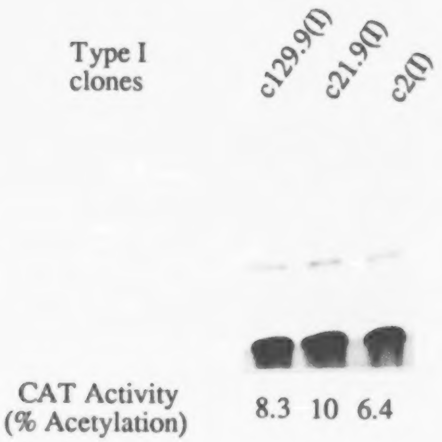


FIGURE IIA.1 Levels of RSV-directed *cat* activity in three independent quiescent type I T cell clones using the adapted DEAE dextran protocol. Clone 129.9(I), 2(I) and 21.9(I) or 85 were produced from CBA/Balb/c mice as described in Havele et al., 1986. The cells were stimulated every 7 days with IL2 (20 units/ml) and antigen (irradiated Balb/c spleen cells). Quiescence was achieved by seeding the cells in low IL2 (10 units/ml) medium for one week. 10^7 cells were transfected with 15 μg of pRSVcat and 500 μg of DEAE dextran in 1 ml of transfection buffer for 5 minutes at room temperature. The cells were then washed in serum-free medium and incubated for 48 hours at 37°C before *cat* determination. An equivalent of 40 μg of protein was used in each case.

medium supplemented with 20 units/ml IL2 for 20 hours. 1.2 x 10^7 activated cells were transfected with 15 µg of pRSVcat and 500 µg DEAE dextran in 1 ml of transfection buffer for 5 minutes at room temperature. Following washes and a 48 hour incubation at 37°C in 5% CO_2 atmosphere, *cat* assays were performed. The level of RSV-driven *cat* activity measured for the quiescent and activated samples are shown in Figure IIA.2. A 5 fold enhancement in *cat* gene expression denoted by an increase in enzyme activity was observed following stimulation of the T cells with antigen and IL2 (7.2% versus 34%). This result agrees with previous reports which have indicated that the RSV promoter contain sequences that respond to antigen and IL2 stimuli or agents that mimick the signals that emanate from these two receptors (Lake et al., 1990, Novak et al., 1990, Siebenlist et al., 1986). Thus, the adapted DEAE dextran transfection method can be applied in cases where T cell activation events are under investigation.

3. *Transfection of type I cells stimulated with PMA and dibutyryl cAMP*

The combination of PMA and dibutyryl cAMP can substitute for the signal normally provided by antigen to elicit a cytotoxic reaction. Type 1 cells (day 9 post-antigen stimulation) were seeded in low IL2 (10 units/ml) medium for 48 hours to force them into quiescence. These cells were then collected and used for transfections at 2 x 10^7 cells/sample. Two series of samples were prepared: the first corresponded to the quiescent cells and the second were stimulated with PMA and dibutyryl cAMP. Trial experiments were performed to determine the optimal concentration of inducers to use for type I cells without compromising cell viability. We found that cell viability was not affected up to 1000 µM of dibutyryl cAMP (stock 100 mM) in the presence of IL2 (30 units/ml) and PMA at a concentration of 15 ng/ml (usual working concentration; stock 10 µg/ml)). We therefore chose to use 700 µM dibutyryl cAMP as an arbitrary concentration for stimulating the cells following transfection. Adsorption of the pRSVcat DNA/DEAE dextran cocktail was set for either 5 or 15 minutes at room temperature. The cells were washed and

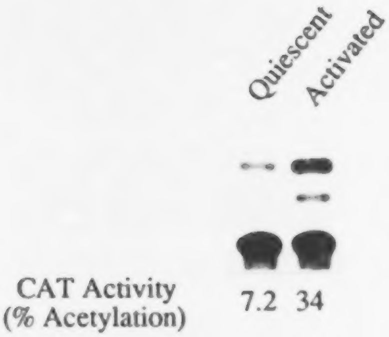


FIGURE IIA.2 Levels of *cat* activity in type I T cell clone 85 following stimulation with antigen and IL2. Quiescent cells were obtained by seeding the cells in low IL2 medium (2 units/ml) for 24 hours. Stimulation with antigen and IL2 was performed as described in Havele et al., 1986 (see text). 1.2 x 10^7 quiescent or activated cells were transfected with 15 µg pRSVcat and 500 µg of DEAE dextran in 1 ml of transfection buffer for 5 minutes at room temperature. Cells were washed and further incubated at 37°C for 48 hours before harvest for *cat* assays.

dispensed into five 100 mm plates containing complete medium supplemented with 20 units/ml of IL2. Stimulation was set at 19 hours post-transfection with 15 ng/ml of PMA and 700 μM dibutyryl cAMP. Cells were collected 27 hours post-stimulation and *cat* activity was measured. Figure IIA.3 shows the level of RSV driven-*cat* activity obtained in the three samples examined, the quiescent cells and the PMA/dibutyryl cAMP-activated cells transfected for 5 or 15 minutes at room temperature. An increase in the *cat* gene expression was observed following activation of the type I cells. The enhancement was greater when the cells were transfected for 5 minutes than for 15 minutes (5 fold versus 2 fold). The same amount of protein was used in each case yet the signal in activated cells are quite different.

A comparison of Figure IIA.1 with Figure IIA.2 reveals that both types of stimulation i.e antigen/IL2 or PMA/dibutyryl cAMP/IL2 result in the same level of *cat* gene expression. The signals transduced by these inducers appear to affect the RSV promoter in a very similar manner leading to the stimulation of the *cat* gene transcription.

In summary, the DEAE dextran transfection protocol optimized for type II cytotoxic T cells can be used successfully and reproducibly on type I cytotoxic T cell lines. Their cell viability and efficiency of transfection are best when the adsorption step is set for only five minutes at room temperature. The development of this adapted technique will facilitate the study of the mechanisms of gene activation in systems such as quiescent and stimulated T cell lines.

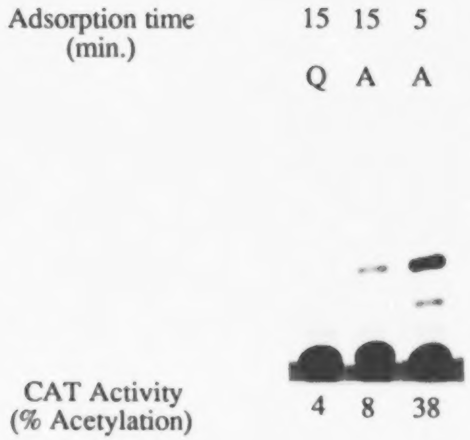


FIGURE IIA.3 Levels of *cat* activity in type I T cell clone 85 following PMA and dibutyryl cAMP stimulation and different adsorption times. Quiescent cells were obtained by seeding the cells in low IL2 medium (10 units/ml) for 48 hours. These cells were used for transfection at a concentration of 2 x 10^7 cells per sample. Adsorption times were set for 5 or 15 minutes at room temperature in the presence of 15 µg of pRSVcat and 500 µg of DEAE dextran in 1 ml of transfection buffer. The cells were then washed twice and placed in complete medium at 37°C in a 5% CO_2 atmosphere. Stimulation was set 19 hours post-transfection using 15 ng/ml of PMA and 700 µM of dibutyryl cAMP. The cells were collected for *cat* determination 27 hours post-stimulation. Q: quiescent, A: activated.

BIBLIOGRAPHY

Fujita, T., Shibuya, H., Ohashi, T., Yamanishi, K. and Taniguchi, T. (1986). Regulation of human interleukin-2 gene: functional DNA sequences in the 5' flanking region for the gene expression in activated T lymphocytes. *Cell* **46**:401-407.

Havele, C., Bleackley, R.C. and Paetkau, V. (1986). Conversion of specific to nonspecific cytotoxic T lymphocytes. *J. Immunol.* **137**:1448-1454.

Lake, R.A., Wotton, D. and Owen, M.J. (1990). A 3' transcriptional enhancer regulates tissue-specific expression of the human CD2 gene. *EMBO J.* **9**:3129-3136.

Novak, T.J., Chen, D. and Rothenberg, E.V. (1990). Interleukin-1 synergy with phosphoinositide pathway agonists for induction of interleukin-2 gene expression: molecular basis of costimulation. *Mol. Cell. Biol.* **10**:6325-6334.

Siebenlist, U., Durand, D.B., Bressler, P., Holbrook, N.J., Norris, C.A., Kamoun, M., Kant, J.A. and Crabtree, G.R. (1986). Promoter region of interleukin-2 gene undergoes chromatin structure changes and confers inducibility on chloramphenicol acetyltransferase gene during activation of T cells. *Mol. Cell. Biol.* **6**:3042-3049.

CHAPTER III

TRANSCRIPTION OF TWO CYTOTOXIC CELL PROTEASE GENES IS UNDER THE CONTROL OF DIFFERENT REGULATORY ELEMENTS[1]

A. INTRODUCTION

When T lymphocytes emerge from the thymus, they have the potential to become either regulators or effectors of cell mediated immune responses. In order to realize their potential the virgin cells must first be stimulated by antigen and interleukin 2 (IL2). This final stage of activation is dependent on the transduction of signals from these receptors into the nucleus resulting in the transcriptional activation of genes that encode function-related proteins. Over the past few years a number of second messengers have been implicated in the activation of T lymphocytes. These include changes in membrane potential, calcium fluxes, phospholipids, kinases, diacylglycerol and cAMP (reviewed in *Immunological Reviews* **95**, 1987). However, the majority of these studies have been correlative in nature and it has been difficult to establish a cause and effect relationship.

Rather than studying membrane associated events we have focussed on the transcriptional activation that occurs upon lymphocyte stimulation. Such an approach has led to the identification of a number of DNA sequences that act as binding sites for regulatory proteins that respond to the membrane signals generated after T lymphocyte stimulation [reviewed by Crabtree (1)]. Most of these studies have focussed on helper T cells, while little work has been done on cytotoxic T lymphocytes. There is, however, evidence that the mechanisms of signal transduction/second messengers are different for activation of the two cell types (2,3,4) and obviously a different set of genes are induced in each case.

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[1] A version of this chapter has been accepted for publication in *Nucleic Acids Research*.

Upon cytotoxic T cell activation a number of genes are induced that encode function related proteins, including perforin (5), lipase (6) and a family of serine proteases (7,8,9). These proteins are packaged within the cytoplasmic granules that are believed to deliver the lysis effector molecule(s) to the target cell (reviewed in *Immunological Reviews* **103**, (9)). The events that control the expression of these different genes remain to be elucidated. The cytotoxic cell protease (CCP) genes (9) are particularly interesting in this regard as they map to the same region of chromosome 14 close to the TCR α locus (10) where another related protease gene encoding cathepsin G has now been localized (11). In contrast to the CCP genes that are expressed in cytotoxic cells, cathepsin G transcription is restricted to myeloid lineage cells (12). Thus this clustered family of protease genes must be subject to different control mechanisms in order to achieve cell-specific expression.

The genomic versions of two of the serine protease genes, C11 and B10 that encode CCP1 (granzyme B) and CCP2 (granzyme C) respectively, were recently cloned and their sequences compared (13,14). A number of potential regulatory regions were identified that were known to play a role in controlling the expression of other T lymphocyte genes. In addition hypersensitive sites were mapped in the 5'-flanking regions of the two genes (13). Here we describe the search for DNA sequences that actually function to regulate the expression of these two genes in cytotoxic T cells using reporter gene constructs transfected into various cell types.

B. MATERIALS AND METHODS

1. *Cells and tissue culture*

The cytotoxic T cell line MTL 2.8.2 was generated from CBA/J mice as described (15). CTLL is a mouse IL2 responsive T cell line but antigen independent (16). EL4.E1 is a murine T lymphoma and an IL2 producing cell line (17). D10 is a mouse helper T cell line (kindly provided by K. Bottomly, Yale). L is a standard mouse fibroblastic cell line. All cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 μM β-mercaptoethanol (RHFM). For MTL 2.8.2 and CTLL, 30 units/ml of IL2 was also included.

2. *Plasmid construction*

Various plasmids were used to generate the constructs. Both pGEM1cat and pGEM2cat were obtained by inserting the BamHI-HindIII 1.6 kb chloramphenicol acetyltransferase (*cat*)-Splice-Polyadenylation cartridge of pSV2cat (18), blunt-ended using *E. coli* Klenow polymerase, into the PvuII site of pGEM1 and pGEM2 vectors (Promega Corporation). pFCΔ56 was provided by W. Leonard (NIH) and contains the mouse c-fos essential promoter sequences, defined by Gilman *et al.* (19), linked to the *cat* gene and downstream SV40-derived sequences from pSVOcat in pUC13. pTKcat was provided by R. Miksicek (Heidelberg) and includes the herpes virus thymidine kinase promoter region -109 to +51 (20) ligated upstream of the *cat* cartridge and polyadenylation site of pSV2cat in pUC8.

The deletion fragments were generated from parental plasmids containing the 5'-upstream region of both B10 and C11 (13), respectively 1648 bp (-1617 to +31) and 1078 bp (-961 to +117) excised from genomic clones with EcoRI-AccI or EcoRI-Tth III1 and subcloned in pUC13. The various fragments were prepared from the parental B10 and C11 plasmids by restriction enzyme cleavage (see Figure III.2). After repair of the ends with either Klenow DNA polymerase or T4 DNA polymerase or both, the fragments were ligated directly to the SmaI site of the promoterless vector pGEM2cat. Only one insert (1080 bp) of B10 was not repaired and inserted directly in the PstI-AccI site of pGEM1cat. For both the pFCΔ56 and pTKcat series, the deletion fragments were inserted into the unique HindIII site of the recipient vectors after addition of HindIII linkers and extensive HindIII digestion. All ligations were performed overnight at 14°C. Plasmid stocks were propagated in *E. coli* strain DH5α and isolated using the alkaline lysis procedure (21). We routinely obtained preparations consisting of more than 90% form 1 (supercoiled) DNA molecules which ensured both consistency and optimal levels of gene expression in transient assays.

A total of seventy constructs were made but only the most informative are presented. A list of these plasmids is given in Table III.1. All constructions were verified either by double restriction digests in the case of the pGEM1cat and pGEM2cat series or by sequence analysis in the case of the pFCΔ56 and pTKcat series. Sequencing was facilitated by the proximity of the inserts in all these latter constructions to the annealing site for the M13 reverse sequencing primer (pFCΔ56) or forward primer (pTKcat). Double stranded sequencing was performed according to Kraft *et al.* (22).

3. *Transfections and cat assays*

MTL 2.8.2 cells were transfected following a DEAE dextran protocol (23) adapted and optimized for cytotoxic cell lines. Basically, 1.25×10^7 cells (taken from cells grown to a density of $3.75\text{-}4 \times 10^6$ cells per 75 cm^2 flask or 1.5×10^5 per ml) were washed twice with serum free medium and resuspended in 1 ml of transfection buffer (0.5 ml of TBS: 25 mM Tris-HCl pH 7.4, 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 0.5 ml of serum free medium) to which 500 µg/ml of DEAE dextran was added (stock: 100 µg/µl made in dH_2O and filter sterilized) then 15 µg of appropriate DNA construct. After DNA adsorption for 15 minutes at room temperature the cells were washed twice with serum free medium. They were finally resuspended in 3 mls of RHFM+ IL2 (30 units/ml), dispensed into three 100 mm plates containing 11 mls of complete medium and were placed at 37°C in 5% CO_2 atmosphere for 48 hours before harvest. The sodium butyrate treatment, where mentioned, was performed as follows: immediately following transfections, MTL 2.8.2 cells were placed in complete medium (RHFM + IL2 (30 units/ml)) supplemented with 7.5 mM sodium butyrate (stock of 500 mM in serum free medium) for 13 hours at 37°C/5% CO_2. The cells were then suspended in fresh medium without drug and further incubated for 35 hours before harvest. CTLL

	Constructs	restricted fragments utilised from the 5'-flanking region	genomic position of the 5'-fragments
B10:	p1648cat	EcoRI-AccI	-1617 to +31
	p1080cat	PstI-AccI	-1049 to +31
	p800cat	HincII-AccI	-769 to +31
	p310cat	BglII-AccI	-279 to +31
	p220cat	HaeIII-AccI	-189 to +31
	p580cat	AluI-AluI	-713 to -133
	p770cat	PstI-BglII	-1049 to -279
	p336cat	PstI-AluI	-1049 to -713
	p568cat	EcoRI-PstI	-1617 to -1049
C11:	p896cat	TaqI-AvaII	-828 to +68
	p495cat	HaeIII-AvaII	-427 to +68
	p311cat	AluI-AvaII	-243 to +68
	p180cat	HinfI-AvaII	-112 to +68
	p439cat	AluI-AluI	-682 to -243
	p184cat	HaeIII-AluI	-427 to -243
	p437cat	HaeIII-HaeIII	-864 to -427
	p255cat	AluI-HaeIII	-682 to -427
	p220cat	AluI-AluI	-902 to -682

TABLE III.1 Summary of the B10 and C11 5'-end-containing plasmids. Full length B10 and C11 5'-upstream regions were cleaved with the specific restriction enzymes indicated and the various fragments cloned into three distinct *cat* plasmids: pGEMcat, pFCΔ56 and pTKcat after polishing the ends with Klenow and/or T4 DNA polymerase and the addition of HindIII linkers as detailed in the Materials and Methods section. The position of the fragments denoted is in reference to the transcriptional start site +1.

transfections followed the MTL 2.8.2 method but only 1 x 10^7 cells per sample were used and dispensed in two 100 mm dishes. Exogenous DNA was introduced in EL4.E1 and D10 cells following the procedure described by Fujita *et al.* (24) but only 1.5 x 10^7 cells and 1 x 10^7 cell per sample were utilized. L cells were transfected using the protocol outlined by Aubin *et al.* (25) using 11% DMSO for 1.5 min at room temperature. In order to establish that the same number of copies of DNA were introduced into each cell line a modified Hirt protocol (26) was used.

Cells were harvested and *cat* assays were performed (18). The protein concentration in the lysates was determined by the Bio-Rad protein assay. An equal amount of protein (usually between 100-200 μg) from each culture was incubated with 0.014 μCi of 45 mCi/mmol, 0.1 mCi ml [^{14}C]chloramphenicol (Dupont/NEN products), 4 mM acetyl coenzyme A (Pharmacia) and 0.5 M tris-HCl pH 7.8 in a final volume of 100-150 μl at 37°C for 4 hours. Reaction products were visualized by autoradiography at room temperature and the relative increases in *cat* activity were determined by liquid scintillation quantification of the acetylated and unacetylated [^{14}C]chloramphenicol after thin layer chromatography. Each series of *cat* assays were performed a minimum of three times and within each set of experiments the parent vectors (pGEMcat, pFCΔ56 and pTKcat) were done in triplicate.

C. RESULTS

1. *The 5'-flanking regions of B10 and C11 control CTL-specific transcription*

Fragments of C11 and B10 corresponding to residues -828 to +68 (896 bp) and -1617 to +31 (1648 bp) respectively (13) were inserted upstream of the *cat* gene in pGEM2cat. The reporter gene in these plasmids does not have its own promoter and thus requires one to be provided by the inserted DNA. Covalently closed circular recombinant plasmid DNA isolated by the method of Birnboim (21) was transfected into recipient cells as described in the Materials and Methods. After the cells were harvested and lysed, *cat* activity was determined.

Autoradiograms obtained with material from a variety of different cell types are shown in Figure III.1. The numbers on the left hand side of each panel refer to the *cat* activity relative to that given by transfection with pGEM2cat. These fragments, corresponding to the 5'-flanking sequences of C11 and B10, result in levels of *cat* activity

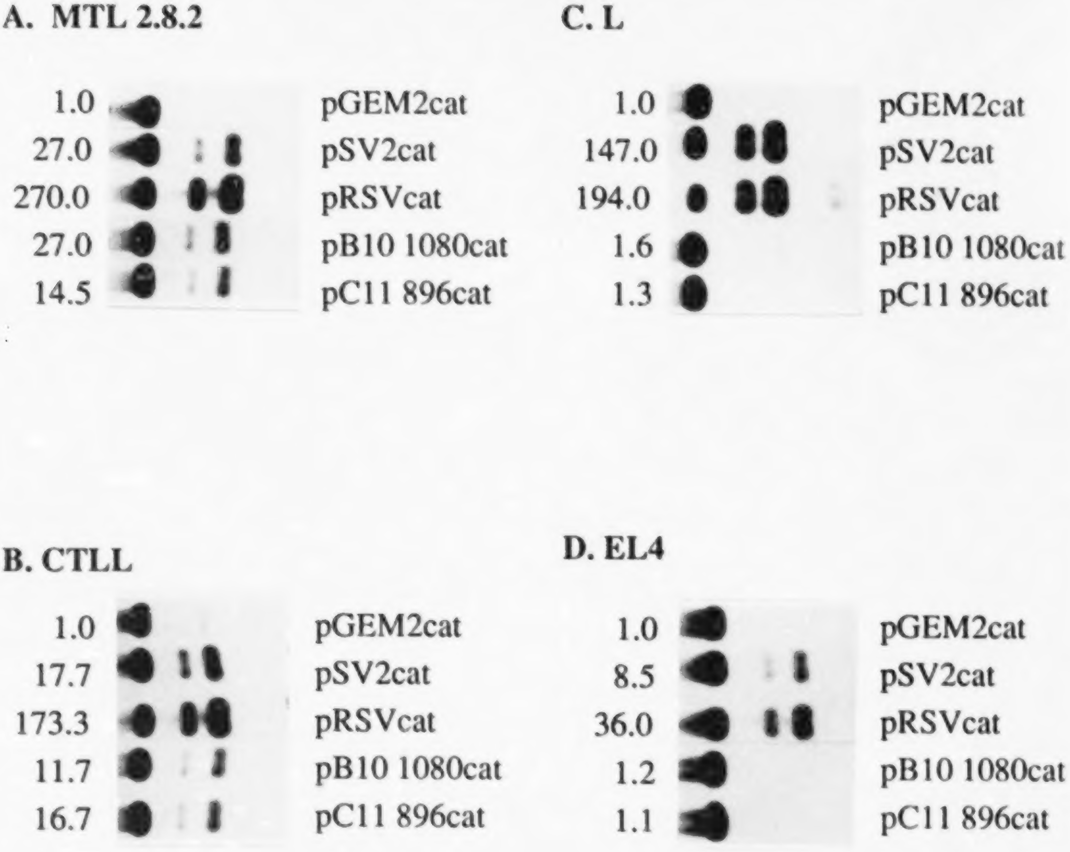

A. MTL 2.8.2
1.0 pGEM2cat
27.0 pSV2cat
270.0 pRSVcat
27.0 pB10 1080cat
14.5 pC11 896cat
C. L
1.0 pGEM2cat
147.0 pSV2cat
194.0 pRSVcat
1.6 pB10 1080cat
1.3 pC11 896cat
B. CTLL
1.0 pGEM2cat
17.7 pSV2cat
173.3 pRSVcat
11.7 pB10 1080cat
16.7 pC11 896cat
D. EL4
1.0 pGEM2cat
8.5 pSV2cat
36.0 pRSVcat
1.2 pB10 1080cat
1.1 pC11 896cat

FIGURE III.1 Cell-specific transcriptional regulation of B10 and C11 5'-flanking regions. Fragments of DNA corresponding to the upstream regions of B10 and C11 genes were ligated upstream of the chloramphenicol acetyltransferase (*cat*) gene and transfected into cytotoxic T cells, MTL2.8.2 and CTLL (panels A and B), L cells (panel C) and EL4 helper T cells (panel D). The constructs used are given on the right of each panel and the *cat* activities relative to the parent plasmid on the left. The absolute values obtained for pGEM2cat and normalized to 1.0 chloramphenicol acetylation corresponded to 0.2% for MTL2.8.2 and L cells and 0.3% for CTLL and EL4. pSV2cat and pRSVcat are included as internal controls.

significantly above that of the promoterless parent plasmid in two CTL clones MTL2.8.2 (panel A) and CTLL (panel B).

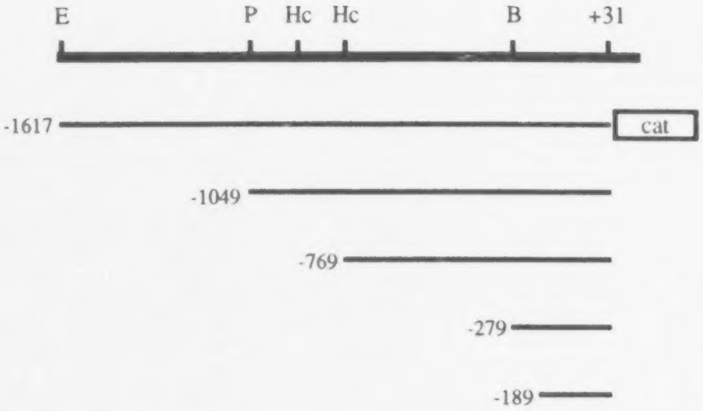
Introduction of these constructs into EL4 cells (panel D) resulted in essentially no reporter activity. The positive results with the two control plasmids, pSV2cat and pRSVcat, clearly indicate that these helper thymoma cells are transfectable. Hirt analyses also revealed the presence of transfected DNA in the nucleus (data not shown). Transfection of another helper T cell clone D10 (data not shown) gave a similar negative result for B10 but a small amount of *cat* for C11 (1.3 times parent). This result agrees with Northern blot analysis on D10 which revealed no expression of B10 but a low level of C11 transcripts (R.C. Bleackley, unpublished). In order to extend our cell specificity studies to non-lymphoid cells the same constructs were introduced into murine L cells. The results shown in panel C are essentially identical to those found for the helper cells i.e. no activity with either B10 or C11 5'-flanking regions. Thus it would appear that the 5'-flanking sequence of B10 and C11 can confer transcriptional competence to the *cat* gene in cytotoxic T cells but they do not function well in T helper cells or non-lymphoid cells.

2. *Deletion analysis of 5'-flanking regions*

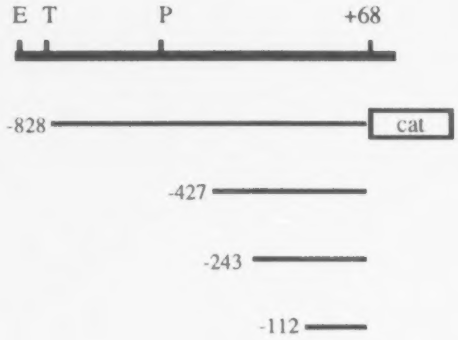
In order to define more closely the promoter elements of the C11 and B10 genes a series of truncated fragments of the 5'-regions were ligated upstream of *cat* and transfected into different cell types. The results are presented in Figure III.2 relative to the *cat* activity given by the parent pGEMcat vector.

On the left hand side the various fragments of either B10 or C11 that were ligated into pGEMcat are indicated with the relative activities on the right. The 5'-flanking region of B10, described as construct p1080cat in Figure III.1, again showed the cell-specific stimulation of activity. Somewhat surprisingly when a longer fragment of B10 was used (construct p1648cat) a lower level of *cat* activity was observed, but the specificity of expression was still retained. As smaller fragments of B10 upstream sequences were

FIGURE III.2 Deletion analysis of B10 and C11 5'-flanking regions. Fragments of the 5'-flanking sequences of B10 (upper panel) and C11 (lower panel) were ligated upstream of the *cat* gene and transfected into a variety of cell types indicated on the right hand side. Numbers presented are *cat* activities relative to the parent pGEMcat plasmid that contains no promoter sequences. Absolute values of chloramphenicol acetylation normalized to 1.0 were for MTL2.8.2 and L cells: 0.2%; EL4 and CTLL cell lines: 0.3% and D10: 0.5%. Each value represents the average of three or more independent experiments which agreed within 8-10%. Abbreviations for the restriction enzyme cleavage sites are E: EcoRI, P: Pst1, Hc: HincII, B: BglII, T: Taq1.


E
P
Hc
Hc
B
+31
-1617
cat
-1049
-769
-279
-189

B10	MTL	CTLL	L	EL4	D10
p1648cat	8.5	5.7	1.1	0.8	0.9
p1080cat	27	12	1.6	1.2	1.0
p800cat	26	12	1.1	1.0	1.2
p310cat	24	19	1.1	0.8	1.0
p220cat	10	4.8	1.1	0.8	1.1


E
T
P
+68
-828
cat
-427
-243
-112

C11	MTL	CTLL	L	EL4	D10
p896cat	15	17	1.3	1.1	1.3
p495cat	11	19	1.5	1.3	1.0
p311cat	18	21	1.5	1.1	1.2
p180cat	1.3	1.9	1.0	0.8	1.0

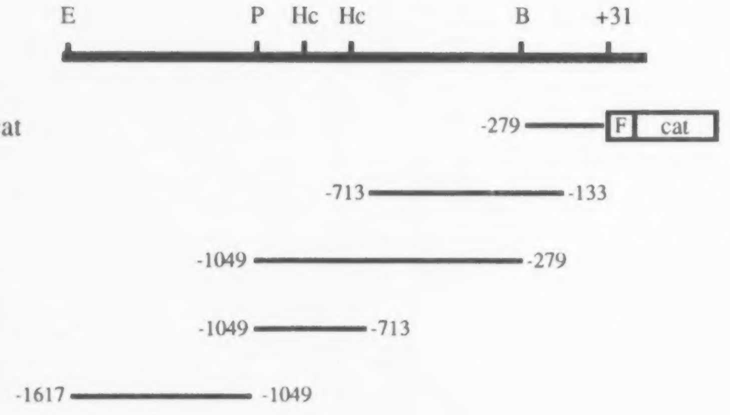
inserted, no large change in activity was observed down to p310cat, but a significant decrease was found in both MTL (2.5 fold) and CTLL (4 fold) between constructs p310cat and p220cat. Clearly a positive regulatory element has been lost, but it should be noted that p220cat can still confer significant CTL-specific activity.

The pattern for C11 is more complicated and appears to involve a number of positive and negative regulatory sequences. The parent C11 plasmid p896cat mediates the CTL-specific expression of *cat* activity described earlier. Deletion of 400 base pairs from the 5'-end of this fragment (construct p495cat) resulted in decrease in *cat* activity in MTL but an increase when introduced into CTLL. Further deletion to yield p311cat resulted in an increase in the activity in both MTL and CTLL. Although the differences in the *cat* signals between p896cat, p495cat and p311cat do not appear significant, other experiments support the presence of regulatory elements within these boundaries (see Figures III.3 and III.5). This same pattern was observed more clearly in MTL2.8.2 when the transfections were performed in the presence of sodium butyrate; p896cat, p495cat and p311cat giving 44, 20 and 35 fold stimulation above pGEM2cat parent. It would appear that residues -243 to +68 of C11 can give wild type levels of activity but both positive and negative elements upstream modulate this depending on the occupancy of these counteracting sites. A further deletion from position -243 to -112 resulted in a dramatic decrease in the level of *cat* activity (14 fold) revealing either the presence of a strong positive element between -243 and -112 or the deletion of a basal promoter element.

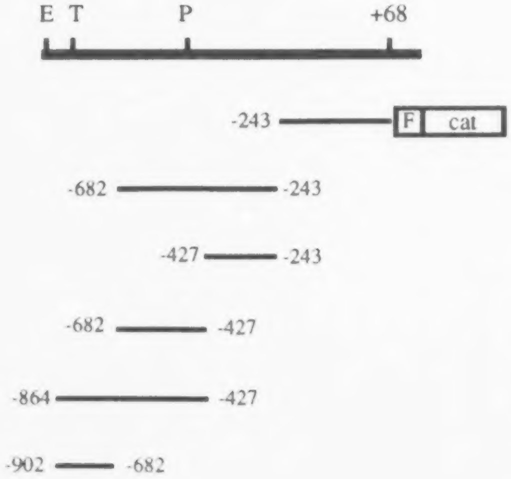
3. *The 5'-regions of B10 and C11 contain elements capable of modulating the fos promoter*

The effect of various fragments of B10 and C11 upstream sequences on the fos promoter are shown in Figure III.3. The basal level of pFCΔ56 obtained for each cell line varied from one fold (EL4) to approximately six fold (MTL, CTLL, L) that given by the promoterless vector pGEM2cat. This value was normalized to 1 and used as the denominator for the determination of relative *cat* activities of the other constructs. The only

FIGURE III.3 Stimulation and repression of transcription by the 5'-flanking sequences of B10 and C11 genes on the *fos* promoter. Fragments of the upstream regions of B10 (upper panel) and C11 (lower panel) genes were ligated upstream of the minimal *fos* promoter in pFCΔ56 (indicated on the left hand side) and transfected into a variety of cell types. The recipient cells and the *cat* activities relative to the parental pFCΔ56 plasmid are shown on the right. The absolute values for pFCΔ56 driven *cat* activities for each cell line were the following: MTL2.8.2, 1.0%; CTLL, 2.0%; L, 1.6%; EL4 and D10, 0.2%. Results are the mean of triplicate or more experiments and standard deviations were between 5-10%. For abbreviations see Figure III.2.


E
P
Hc
Hc
B
+31
-279
F
cat
-713
-133
-1049
-279
-1049
-713
-1617
-1049

B10	MTL	CTLL	L	EL4	D10
p310FOScat	5.7	4.7	2.5	3.0	1.5
p580Fcat	0.3	0.9	0.2	1.0	1.0
p770Fcat	0.2	0.8	0.2	1.0	1.0
p336Fcat	0.3	0.8	0.1	1.0	1.0
p568Fcat	0.2	0.3	0.2	0.6	1.0


E
T
P
+68
-243
F
cat
-682
-243
-427
-243
-682
-427
-864
-427
-902
-682

C11	MTL	CTLL	L	EL4	D10
p311FOScat	1.9	2.3	0.1	0.5	2.0
p439Fcat	13	6.9	5.1	11	24
p184Fcat	0.8	2.0	0.9	1.5	1.0
p255Fcat	0.5	0.6	0.2	0.6	1.0
p437Fcat	0.5	0.5	0.5	1.0	1.0
p220Fcat	0.3	0.5	0.3	0.5	0.5

B10 5'-sequence that gave a major increase in *cat* activity above the basal level seen with the parental fos promoter plasmid was p310Fcat. This is the same fragment of DNA that gave a 24 fold increase in activity in the promoterless vector assay described earlier. None of the other 5'-flanking fragments endowed the fos promoter with any significant enhancement of activity, indeed they appeared to suppress *cat* expression. Most interestingly, the far upstream region of B10 (-1617 to -1049) by itself appeared to have a negative effect on fos-driven *cat* gene expression. This result confirms the presence of a negative modulatory element within that region implied in Figure III.2 by comparison of p1648cat with p1080cat.

The results observed with the fos promoter in the non-CTL cells were interesting as this same fragment of DNA corresponding to residues -279 to +31 of B10 also gave an increase in *cat* activity. In the promoterless pGEM2cat vector expression was observed exclusively in cytotoxic cell lines. Thus we have the situation of a positive upstream regulatory element that functions on a heterologous but not the normal promoter in non-expressing cells. Either the B10 promoter is suppressed in the non-CTL or these cells are missing a factor(s) necessary for promoter activity.

The 5'-flank of C11 was also analyzed using the pFCΔ56 reporter plasmid. A significant enhancement of *cat* activity was seen with construct p439Fcat in both CTL lines. This fragment corresponds to part of the region of DNA that was lost between p896cat and p495cat described in the lower panel of Figure III.2. In those experiments the MTL transfection results indicated the presence of a positive regulator. The two plasmids p184Fcat and p255Fcat cover the whole of p439Fcat but neither give significant stimulation of *cat* activity above the pFCΔ56. This would indicate that the binding site for the regulatory protein in question is very close to the HaeIII site at -427 of C11. Alternatively, this may indicate the presence of separate regulatory sequences on these two fragments that act synergistically, but not independently. As with the B10 positive element contained in pB10 310Fcat, the pC11 439Fcat gave significant levels of activity in L, EL4 and D10 cells.

The other fragment that showed high levels of *cat* activity in the C11 promoter deletion experiment (i.e. p311cat construct comprising an insert corresponding to residues -243 to +68) stimulated the activity on the fos promoter (p311Fcat, Figure III.3) slightly but nowhere near the 20 fold level seen in Figure III.2. Thus the two positive regulatory elements defined with the natural promoter appear to act differently in magnitude with this heterologous promoter.

In all three cases, p310Fcat, p439Fcat and p311Fcat, the modulatory elements appeared to work as well in the opposite orientation giving rise to similar levels of *cat* activity (see Appendix to Chapter III). These observations exclude the possibility that our results could be explained by the additive or competitive effect of the two promoters in tandem as, in this orientation, the B10 and C11 promoters could not influence the fos promoter-dependent transcription of the *cat* reporter gene.

4. *Tissue specificity of homologous versus heterologous promoters*

We then directly compared the ability of the whole 5'-flanking regions versus the individual positive elements of B10 and C11 to mediate tissue-specific expression when fused to the fos promoter. The results are shown in Figure III.4. As previously the *cat* activities were normalized to the parent pFOScat transfectants. For B10 p1080Fcat and p310Fcat gave significant stimulation of activity in both MTL and CTLL. However while the isolated positive element mediated transcription in L and EL4 cells the larger 5'-fragment retained its tissue specificity and did not increase the level of *cat* above the parent. The result for C11 is very similar. Constructs pC11 896Fcat (large fragment of 5'-flanking region) and pC11 439Fcat (positive element) both gave expression in the cytotoxic cells but only pC11 439Fcat was able to stimulate expression in the non-CTL cells. Thus for both

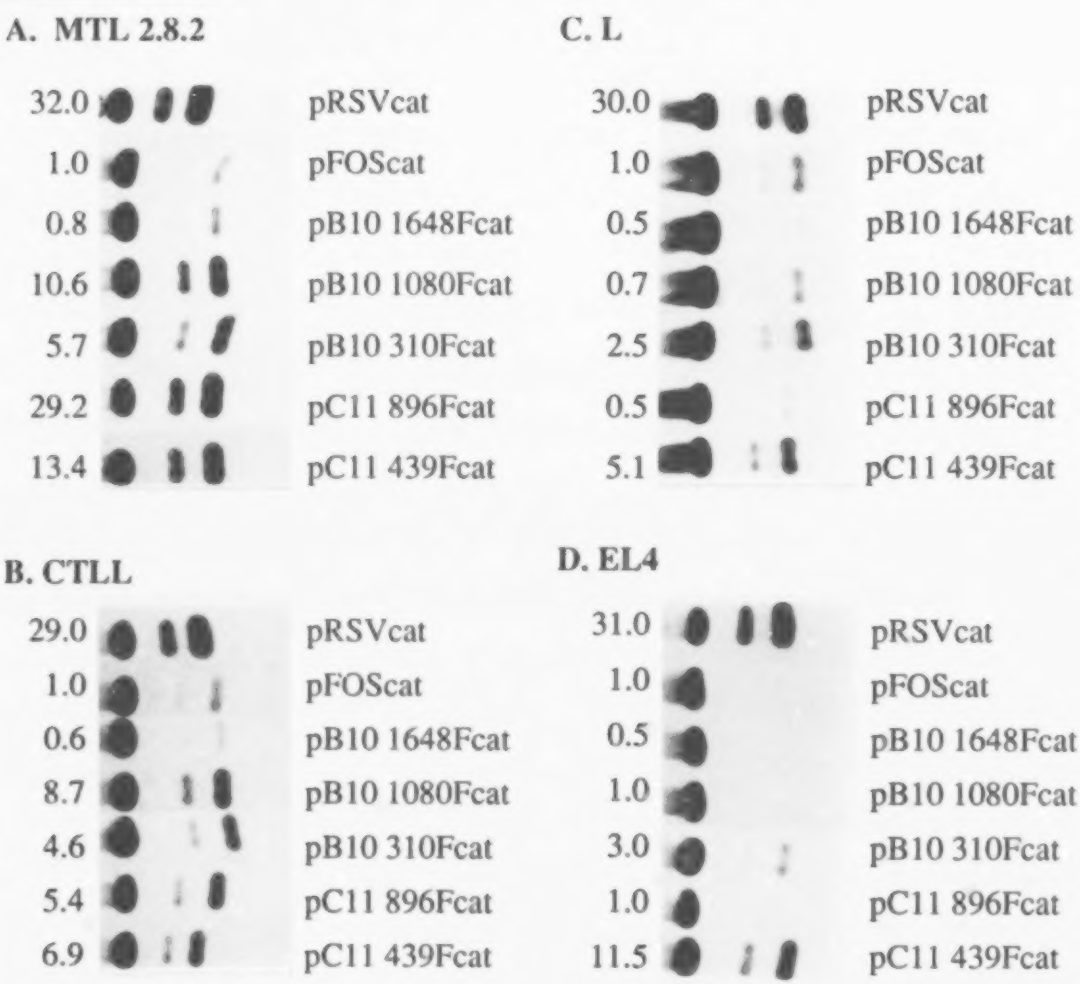

A. MTL 2.8.2
32.0 pRSVcat
1.0 pFOScat
0.8 pB10 1648Fcat
10.6 pB10 1080Fcat
5.7 pB10 310Fcat
29.2 pC11 896Fcat
13.4 pC11 439Fcat
C. L
30.0 pRSVcat
1.0 pFOScat
0.5 pB10 1648Fcat
0.7 pB10 1080Fcat
2.5 pB10 310Fcat
0.5 pC11 896Fcat
5.1 pC11 439Fcat
B. CTLL
29.0 pRSVcat
1.0 pFOScat
0.6 pB10 1648Fcat
8.7 pB10 1080Fcat
4.6 pB10 310Fcat
5.4 pC11 896Fcat
6.9 pC11 439Fcat
D. EL4
31.0 pRSVcat
1.0 pFOScat
0.5 pB10 1648Fcat
1.0 pB10 1080Fcat
3.0 pB10 310Fcat
1.0 pC11 896Fcat
11.5 pC11 439Fcat

FIGURE III.4 Cell-specific regulation conferred by 5'-flanking sequence of B10 and C11 upstream regions. Fragments of B10 and C11 5'-flanking sequences (described in Figures III.2 and III.3) were ligated upstream of the *fos* promoter in pFCΔ56 and transfected into cytotoxic T cells (MTL2.8.2, panel A, and CTLL, panel B), a non-lymphoid cell line (L cells, panel C), and a helper T lymphoma (EL4, panel D). Constructs are indicated on the right hand side and *cat* activities relative to the parent pFCΔ56 are shown on the left of each panel. For absolute values of *cat* activity for the parent plasmid pFCΔ56 see Figure III.3.

of these positive elements within the context of their natural environments, they provide cell-specific regulatory control; however, in isolation specificity is lost.

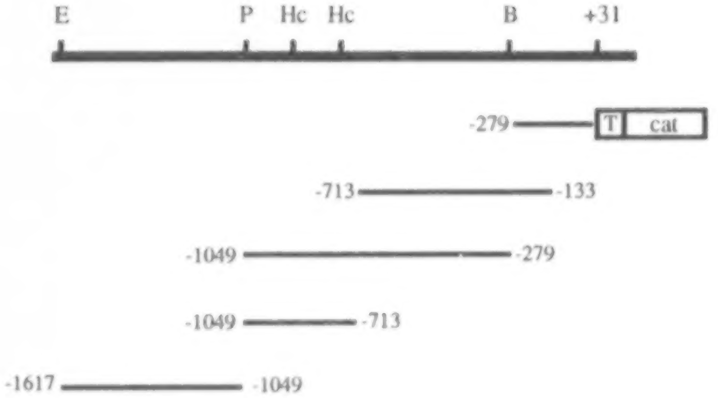
5. *Do B10 and C11 regulatory regions work on the tk promoter?*

We next asked whether any of the positive and negative elements uncovered so far would function on the thymidine kinase promoter. The basal level of this promoter is six fold higher than that of fos, thus the effects of positive regulatory elements can be masked due to the strength of the tk promoter. Despite this we saw a small but significant enhancement of activity with constructs pB10 310TKcat and pC11 311TKcat as shown in Figure III.5. Surprisingly the dominant positive regulatory element revealed for C11 in the fos promoter experiments appeared to suppress transcription from the tk promoter. In addition, the 568 bp fragment which behaved as a negative element in the context of its own promoter and the fos promoter did not seem to act as such when placed immediately upstream of the tk promoter. As for the fos promoter, the fragments inserted upstream of the tk promoter worked in both orientations, thus obviating the possibility that our results are due to a summation or interference of transcription from two promoters.

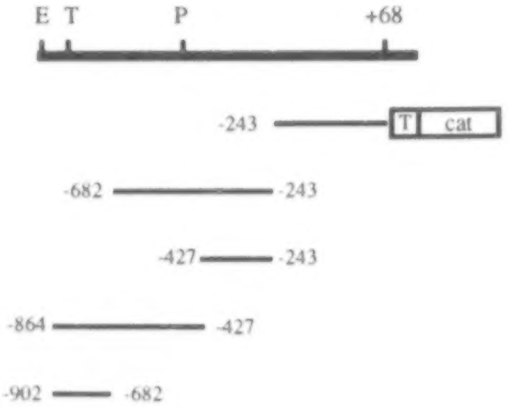
D. DISCUSSION

During cytotoxic T lymphocyte activation a number of genes become transcriptionally active including those encoding perforin, lipase and a family of proteases. The proteases are particularly interesting as, in the mouse, there are at least six closely related members, four of which are encoded in the same region of chromosome 14 (10). Thus it was believed that all of these protease genes would share a common transcriptional control mechanism. It was therefore unexpected to find when the genomic sequences of two family members C11 and B10 (encoding cytotoxic cell protease 1 (granzyme B) and 2 (granzyme C)) were isolated, that the sequences outside the protein coding regions were quite different (13). Computer-assisted searching for homologous regions was un-

FIGURE III.5 Stimulatory and suppressing activity of 5'-flanking regions of B10 and C11 genes on the thymidine kinase promoter. Fragments of DNA from the upstream regions of B10 and C11 genes (indicated on the left) were ligated upstream of the tk promoter and transfected into a variety of cells (denoted on the right). *Cat* activities relative to the parental pTKcat construct are shown on the right hand of the panel. The absolute values of *cat* activities measured for pTKcat were 5.0% for MTL2.8.2, 4.0% for L cells, 8.0% for CTLL, 0.4% for EL4 and 0.8% for D10. Values represent the mean of three or more independent experiments and standard deviations were between 8-10%. For abbreviations see Figure III.2.


E
P
Hc
Hc
B
+31
-279
T
cat
-713
-133
-1049
-279
-1049
-713
-1617
-1049

B10	MTL	CTLL	L	EL4	D10
p310TKcat	4.0	1.7	0.7	1.3	0.9
p580TKcat	1.4	1.1	0.3	0.8	0.6
p770TKcat	0.7	0.6	0.4	0.8	0.5
p336TKcat	0.6	0.7	0.2	1.0	0.4
p568TKcat	0.8	0.4	0.1	0.5	0.6


E
T
P
+68
-243
T
cat
-682
-243
-427
-243
-864
-427
-902
-682

C11	MTL	CTLL	L	EL4	D10
p311TKcat	2.2	0.6	0.4	0.5	0.5
p439TKcat	0.3	1.1	0.2	0.5	0.4
p184TKcat	0.5	0.6	0.1	0.5	0.5
p437TKcat	0.4	0.6	0.1	0.8	0.4
p220TKcat	0.2	0.7	0.3	0.5	0.3

revealing, although manual visual inspection did identify some similar sequences. A number of these had been implicated as *cis*-acting control elements for other T cell genes. In order to test whether any of these were playing a role in the control of CCP1 and 2 transcription and to identify other unknown sequence elements, a series of constructs was made consisting of various fragments of the B10 or C11 genes fused to the reporter gene *cat*. These were transfected into a variety of cell types.

Transfections with large fragments of either B10 (~ 1100) or C11 (~ 900 base pairs) 5'-upstream sequences in a promoterless vector increased the levels of *cat* activity significantly above those obtained with vector alone in CTL lines. No B10 or C11 driven *cat* expression was seen in either the helper lymphoma line EL4 or murine L cells. In another T helper cell D10, although no activity was detected with B10 sequences, a small amount was seen with the C11 fragment. However, a low level of C11 but no B10 expression has been detected from the endogenous genes in D10 by Northern blot analysis (RCB unpublished).

The conclusion from this first set of experiments is that the 5'-flanking regions of B10 (-1049 to +31) and C11 (-828 to +68) contain positive regulatory elements that are responsible for the CTL-specific expression of the two genes. However, it should be noted that this conclusion is based upon transfections in cells that differ in CD8 expression. We have not evaluated other cytotoxic cells such as NK cells or CD4$^+$ killers thus, we have not excluded the possibility that the differences are related to CD8 expression rather than function. Using a series of deleted upstream fragments these regulatory regions were mapped further.

For the B10 gene the results indicate that a negative element is present between -1617 and -1049 (p1648cat versus p1080cat, Figure III.2) and indeed this fragment suppressed transcription from the c-fos promoter (Figure III.4). The major positive region appears to be contained within construct p310cat (Figure III.2) and this was confirmed when this fragment was ligated upstream of both the fos (Figure III.3) and tk (Figure III.5) promoters. However, a comparison of the activities of p310cat and p220cat in Figure III.2 indicates that a significant reduction has occurred with the deletion of 90 base pairs from the 5' end of p310cat. Thus we conclude that a major positive regulatory element is contained within residues -279 to -189 of the B10 genomic sequence.

The C11 5'-flanking region appears to contain two positive regulatory sequences, one indicated by the decrease in *cat* activity when p896cat and p495cat are compared and the other contained within construct p311cat (Figure III.2 and sodium butyrate data). The former of these was confirmed when the relevant region of DNA was ligated upstream of the fos promoter (construct pC11 439Fcat, Figure III.3). These results suggest that a positive regulatory sequence is contained between residues -682 and -427. The lack of positive activity with both pC11 184Fcat and pC11 255Fcat may indicate that the regulatory protein binding site is close to residue -427. Alternatively insert 439 may contain two binding sites that have to be linked to induce an increase in transcriptional activity.

Similarly, Hanson and Ley (27) have recently mapped a strong positive element within the same region (-682 to -427) of the 5'-flanking sequences of the human homologue of C11. Both human and mouse C11 genes share a high degree of homology in this region. This conserved positive element may thus be important in the transcriptional activation of the C11 gene.

The regions of DNA where we have mapped these regulatory sequences is extremely interesting as they correspond to the sites where we have previously detected DNaseI hypersensitive sites in the 5'-flanking regions of B10 and C11 (13). These sites were found in CTL clones but not in unstimulated thymocytes. Our prediction at the time was that these regions of DNA would be the ligand binding sites for the regulatory proteins that control B10 and C11 expression in activated cytotoxic cells.

Inspection of the sequences in the regions suspected to contain the positive transcriptional regulatory sequence for B10 (-279 to -189) and C11 (-682 to -427 and -243 to -112) revealed the presence of only two known regulatory elements that were shared by the two genes but differed from the consensus motifs. Both of the C11 fragments contain AP1 sites (28) at -565 (TCAGTCAG) and -143 (TGAGTCAT) but the AP1-like motif in the B10 5'-flanking region at -219 (TGCAGTCAT) is different from the consensus sequence and may therefore not function as such. An NFκB-like sequence (29) is located at -230 (GGGACTCTGATACC) in the C11 proximal regulatory region. B10 also has an NFκB-like motif at -210 (GATGGACTTCCT) but its sequence is distinct from the C11 element. The C11 proximal region also includes an AP2 binding site (30) at -203 (CCCCCACCCC), two Moloney murine leukemia virus or SL3-3 leukemogenic virus enhancer core sequences (31) at -185 (TGTGGTCTT) and -126 (TGTGGTTAC), and a CRE (cAMP responsive element, (32)) motif at -91 (AGACGTCA). In addition, the C11 distal regulatory region contains an OCT (octamer binding) site, (33) at -632 (TTTGCATC), an NFAT (nuclear factor of activated T cells)-like purine motif (34) at -604 (AAGAAGTAGGAA) and a CLE1 (conserved lymphokine element 1) site (35) at -566 (AGTGATTCCAC). We conclude therefore that these two genes, that are so similar within their transcribed regions and are clustered together on chromosome 14, are regulated by different *cis*-acting sequences and *vide infra* different regulatory proteins. This would explain our previous observations that B10 and C11, although they are both transcriptionally induced upon CTL activation, are expressed at different levels and with different kinetics (13).

The "whole" 5'-flanking regions of the two genes (B10 1080 bp and C11 896 bp) confer CTL-specific expression on the reporter gene constructs (Figures III.1 and III.4). However, when the individual positive elements were removed from their natural environment and used to regulate transcription from the fos promoter this cell-restricted expression was lost (Figure III.4). Thus the regulated expression of these protease genes is achieved not just by the presence or absence of a particular element but by the protein in a particular environment interacting with other regulatory factors and promoter binding proteins.

This combinatorial aspect of gene regulation is underscored for the regulatory elements described here if we consider the different results achieved with various combinations of positive elements and promoters summarized in Table III.2. The large B10 (1080 bp) sequence works well on its own promoter and with fos but not to a significant degree with the tk promoter. In contrast the isolated B10 positive element found within insert 310 works well with all the promoters. For C11 the full 896 bp fragment functions well by itself or in combination with fos, however when fused to tk it does not function at all. Even more intriguing results were obtained when this fragment was dissected into its two positive elements described in constructs p439cat and p311cat. While the former stimulated fos and suppressed tk, the latter worked reasonably on tk but significantly worse on fos, when compared to the effect of p896cat and p439cat on fos. This discrepancy between the fos and tk series emphasizes the importance of using various promoters with different strengths when searching for regulatory elements in the flanking regions of genes.

Recently the ability of a muscle-specific enhancer to function in conjunction with the myoglobin promoter but not the SV40 promoter has been explained on the basis of differences in TATA binding proteins (36). Similarly the activity of the activation transcription factor of the HSP70 gene was dramatically altered by the TATA motif. In contrast the CP1 factor functioned regardless of the motif (37). Thus in the case of the HSP70 gene both TATA-dependent and TATA-independent regulation is possible. The C11 gene has a motif CATAAAA that closely resembles that of the fos promoter (TATAAA) but is quite different from the equivalent region in the tk gene (ATATTAA). The ability of C11 439 bp fragment to stimulate the fos but not the tk promoter could indicate that it functions in part by interaction with a specific TATA binding protein. In contrast it is not obvious that B10 has a TATA motif, the closest is the sequence CAAAAT. If B10 is indeed TATAless one would expect the B10 310 binding protein to stimulate both tk and fos promoters through the interaction with some other part of the transcriptional

Plasmid/(nucleotides)	Promoters		
	natural	*fos*	tk
pB10 1080 (-1049 to +31)	27	11	1.5
pB10 310 (-279 to +31)	24	6.0	4.0
pC11 896 (-828 to +68)	15	29	0.9
pC11 439 (-682 to -243)	-	13	0.3
pC11 311 (-243 to +68)	18	2.0	2.2

TABLE III.2 5'-flanking regions of B10 and C11 genes stimulate different promoters differently. Fragments of B10 and C11 5'-flanking regions were ligated into pGEMcat, pFCΔ56 or pTKcat and transfected into MTL2.8.2. *Cat* activities were determined and are expressed relative to the parent plasmids. Absolute values for the percentage of chloramphenicol acetylation normalized to 1.0 were for pGEMcat, 0.2; pFCΔ56, 1.0; pTKcat, 5.0. These values represent the average of three or more independent experiments which agreed with 5-10%.

machinery. As shown in Table III.2, this is indeed the case. Clearly the situation is complex and the mere presence of these regulatory elements is not sufficient to determine CTL-specific transcriptional control. The context of the elements and interactions between the proteins they bind must be considered of paramount importance. Obviously further experiments are needed to determine how these elements function and regulate tissue-specific expression during CTL activation.

In summary (see Figure III.6), we have provided the first experimental dissection of the elements that function to control the transcription of two members of the murine cytotoxic cell protease family of genes. Sequences within the 5'-flanking regions of both C11 (~ 900 base pairs) and B10 (~ 1100 base pairs) are sufficient to confer CTL-specific

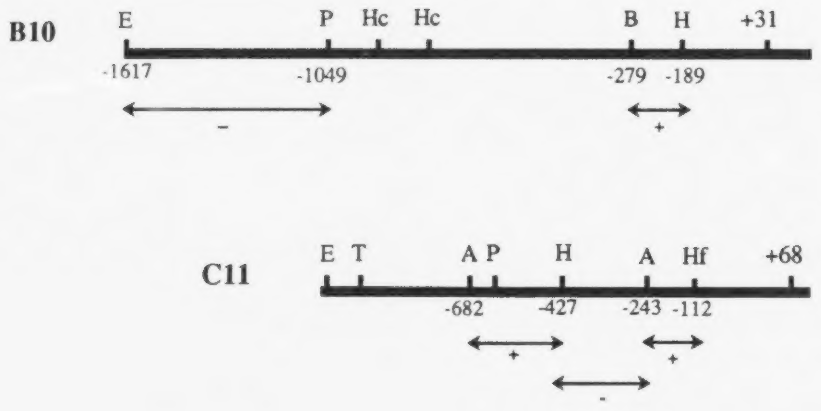

B10
E
P
Hc
Hc
B
H
+31
-1617
-1049
-279
-189
-
+
C11
E
T
A
P
H
A
Hf
+68
-682
-427
-243
-112
+
+
-

FIGURE III.6 Summary of *cis*-acting regulatory elements in the 5'-flanking regions of B10 and C11 genes. Areas indicated with - either downregulate or their deletion results in an increase in transcription. Positive regulatory regions are marked with **+**. Abbreviations for the restriction enzyme cleavage sites are E: EcoR1, P: Pst1, Hc: HincII, B: BglII, H: HaeIII, T: Taq1, A: AluI, Hf: HinfI.

expression on a reporter gene. Further deletion analyses suggest that these regions of DNA contain positive regulatory elements; two for C11 and one for B10 based upon their behavior in CTL lines. These sequences, which correspond to DNase1 hypersensitive sites *in vivo*, can also confer transcriptional stimulatory activity on heterologous promoters but when removed from their natural environments lose their cell specificity. Inspection of the DNA sequences in the regions of the positive regulatory elements reveal that they are different between the two genes and this is also apparent functionally when their effects on different promoters is compared. Finally, the results described here cannot be explained on the basis of any of the previously characterized regulatory elements. Thus the cell-specific regulation of these two genes appears to be under the control of different and novel regulatory elements that achieve their effects within their specific genomic contexts. It will be most interesting to see if this pattern extends to other members of this protease gene cluster including other CCPs and cathepsin G.

BIBLIOGRAPHY

1. Crabtree, G.R. (1989). Contingent genetic regulatory events in T lymphocyte activation. *Science* **243**:355-361.

2. Mills, G.B., Cheung, R.K., Grinstein, S. and Gelfand, E.W. (1985). Increase in cytosolic-free calcium concentration is an intracellular messenger for the production of IL2 but not for expression of the IL2 receptor. *J. Immunol.* **134**:1640-1643.

3. Mills, G.B., Cheung, R.K., Grinstein, S. and Gelfand, E.W. (1985). Interleukin 2-induced lymphocyte proliferation is independent of increases in cytosolic-free calcium concentrations. *J. Immunol.* **134**:2431-2435.

4. Kozumbo, W.J., Harris, D.T., Gromkowski, S., Cerottini, J.C. and Cerutti, P.A. (1987). Molecular mechanisms involved in T cell activation. The phosphatidylinositol signal transducing mechanism mediates antigen-induced lymphokine production but not interleukin 2-induced proliferation in cloned cytotoxic T lymphocytes. *J. Immunol.* **138**:606-612.

5. Lowrey, D.M., Aebischer, T., Olsen, K., Lichtenheld, M., Rupp, F., Hengartner, H. and Podack, E.R. (1989). Cloning, analysis, and expression of murine perforin 1 cDNA, a component of cytolytic T-cell granules with homology to complement component C9. *Proc. Natl. Acad. Sci.* **86**:247-251.

6. Grusby, M.J., Nabair, N., Wong, H., Dick, R.F., Bluestone, J.A., Schotz, M.C. and Glimcher, L. (1990). Cloning of an IL4 inducible gene from cytotoxic T lymphocytes and its identification as a lipase. *Cell* **60**:451-459.

7. Lobe, C.G., Finlay, B., Paranchych, W., Paetkau, V.H. and Bleackley, R.C. (1986). Two cytotoxic T lymphocyte-specific genes encode unique serine proteases. *Science* **232**:858-861.

8. Lobe, C.G., Havele, C. and Bleackley, R.C. (1986). Cloning of two genes which are specifically expressed in activated cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci.* **83**:1448-1452.

9. Bleackley, R.C., Lobe, C.G., Duggan, B., Ehrman, N., Frégeau, C., Meier, M., Letellier, M., Havele, C., Shaw, J. and Paetkau, V. (1988). The isolation and characterization of a family of serine protease genes expressed in activated cytotoxic T lymphocytes. *Immunological Reviews* **103**:5-19.

10. Crosby, J.L., Bleackley, R.C. and Nadeau, J.H. (1990). A complex of serine protease genes expressed preferentially in cytotoxic T lymphocytes is closely linked to the T-cell receptor α- and δ-chain genes on mouse chromosome 14. *Genomics* **6**:252-259.

11. Hanson, R.D., Hohn, P.A., Popescu, N.C. and Ley, T.J. (1990). A cluster of hematopoietic serine protease genes is found on the same chromosomal band as the human α/δ T-cell receptor locus. *Proc. Natl. Acad. Sci.* **87**:960-963.

12. Hanson, R.D., Connolly, N.L., Burnett, D., Campbell, E.J., Senior, R.M. and Ley, T.J. (1990). Developmental regulation of the human cathepsin G gene in myelomonocytic cells. *J. Biol. Chem.* **265**:1524-1530.

13. Lobe, C.G., Shaw, J., Frégeau, C., Duggan, B., Meier, M., Brewer, A., Upton, C., McFadden, G., Patient, R.K., Paetkau, V.H. and Bleackley, R.C. (1989). Transcriptional regulation of two cytotoxic T lymphocyte-specific serine protease genes. *Nucleic Acids Research* **17**:5765-5779.

14. Lobe, C.G., Upton, C., Duggan, B., Ehrman, N., Letellier, M., Bell, J., McFadden, G. and Bleackley, R.C. (1988). Organization of the genes encoding two T cell specific proteases, CCPI and II. *Biochemistry* **27**:6941-6946.

15. Bleackley, R.C., Paetkau, V. and Havele, C. (1982). Cellular and molecular properties of an antigen-specific cytotoxic T lymphocyte line. *J. Immunol.* **128**:758-767.

16. Gillis, S. and Smith, K.A. (1977). Long term culture of tumor-specific cytotoxic T cells. *Nature* **268**:154-156.

17. Paetkau, V., Shaw, J., Ng, J., Elliott, J.F., Meerovitch, K., Barr, P.J. and Bleackley, R.C. (1986). Regulation of lymphokine expression. In *Mediators of Immune Regulation and Immunotherapy*. K. Singhal and N.R. Sinclair, N.R., eds., Elsevier Press. p. 3.

18. Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell Biol.* **2**:1044-1051.

19. Gilman, M.Z., Wilson, R.N. and Weinberg, R.A. (1986). Multiple protein-binding sites in the 5'-flanking region regulate c-*fos* expression. *Mol. Cell. Biol.* **6**:4305-4316.

20. McKnight, S.L. and Kingsbury, R. (1982). Transcriptional control signals of a eukaryotic protein-coding gene. *Science* **217**:316-324.

21. Birnboim, H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acid Res.* **7**:1513-1523.

22. Kraft, R., Tardiff, J., Krauter, K.S. and Leinwand, L.A. (1988). Using mini-prep plasmid DNA for sequencing double-stranded templates with Sequenase™. *BioTechniques* **6**:544-547.

23. Banerji, J., Olson, L. and Schaffner, W. (1983). A lymphocyte-specific cellular enhancer is located downstream of the joining regions in immunoglobulin heavy chain genes. *Cell* **33**:729-740.

24. Fujita, T., Shibuya, H., Ohashi, T., Yamanishi, K. and Taniguichi, T. (1986). Regulation of human interleukin-2 gene. *Cell* **46**:401-407.

25. Aubin, R.J., Weinfeld, M. and Paterson, M. (1988). Factors influencing efficiency and reproducibility of polybrene-assisted gene transfer. *Somatic Cell and Molecular Genetics* **14**:155-167.

26. Hirt, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**:365-369.

27. Hanson, R.D. and Ley, T.J. (1990). Transcriptional activation of the human cytotoxic serine protease gene CSP-B in T lymphocytes. *Mol. Cell. Biol.* **10**:5655-5662.

28. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P. and Karin, M. (1987). Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. *Cell* **49**:729-739.

29. Lenardo, M.J. and Baltimore, D. (1989). NF-κB: a pleiotropic mediator of inducible and tissue-specific gene control. *Cell* **58**:227-229.

30. Imagawa, M., Chiu, R. and Karin, M. (1987). Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. *Cell* **51**:251-260.

31. Boral, A.L., Okenquist, S.A. and Lenz, J. (1989). Identification of the SL3-3 virus enhancer core as a T-lymphoma cell-specific element. *J. Virol.* **63**:76-84.

32. Roesler, W.J., Vanderbark, G.R. and Hanson, R.W. (1988). Cyclic AMP and the induction of eukaryotic gene transcription. *J. Biol. Chem.* **263**:9063-9066.

33. Wirth, T., Staudt, L. and Baltimore, D. (1987). An octamer oligonucleotide upstream of a TATA motif is sufficient for lymphoid-specific promoter activity. *Nature* **329**:174-178.

34. Shaw, J.P., Utz, P., Durand, D.B., Toole, J.J., Emmel, E.A. and Crabtree, G.R. (1988). Identification of a putative regulator of early T cell activation genes. *Science* **241**:202-205.

35. Arai, K., Lee, F., Miyajima, A., Miyatake, S., Arai, N. and Yokota, T. (1990). Cytokines: coordinators of immune and inflammatory responses. *Ann. Rev. Biochem.* **59**:783-836.

36. Wefald, F.C., B.H. Devlin and R.S. Williams. 1990. Functional heterogeneity of mammalian TATA-box sequences revealed by interaction with a cell-specific enhancer. *Nature* **344**:260-262.

37. Taylor, I.C.A. and R.E. Kingston. 1990. Factor substitution in a human HSP70 gene promoter: TATA-dependent and TATA-independent interactions. *Mol. Cell. Biol.* **10**:165-175.

APPENDIX TO CHAPTER III

In order to exclude the possibility that our results could be explained by the additive or competitive effect of the two promoters in tandem, i.e the cellular fos or viral tk fused to C11 or B10, some of the deletion fragments were fused in the antisense orientation to the *cat* reporter gene. The resultant constructs were then introduced in the MTL2.8.2 cells following the adapted DEAE dextran protocol and tested for their ability to direct the expression of the *cat* gene. The levels of *cat* activity obtained are presented in Table IIIA.1.

Constructs	Orientation of B10/C11 5'-Flanking Fragment	% Chloramphenicol Acetylation
pB10 1080Fcat	A	11 +/- 0.8
pB10 1080Fcat	B	9 +/- 0.4
pB10 310Fcat	A	5.6 +/- 0.3
pB10 310Fcat	B	5.2 +/- 0.5
pC11 896Fcat	A	28 +/- 2.2
pC11 896Fcat	B	31 +/- 1.5
pC11 311Fcat	A	1.9 +/- 0.2
pC11 311Fcat	B	2.0 +/- 0.2
pC11 439Fcat	A	13 +/- 1.1
pC11 439Fcat	B	10 +/- 0.6

TABLE IIIA.1 Comparison of the levels of *cat* activity obtained from the c-fos/*cat* constructs containing the 5'-flanking fragments of C11 and B10 in the sense ("A") or antisense ("B") orientation. Restricted fragments derived from the 5'-flanking region of C11 and B10 were fused in the "A" or "B" orientation upstream of the *cat* cassette in the pFC Δ56 vector. Following transfection in MTL2.8.2 cells as described in Materials and Methods, the ability of each construct to direct the transcription of the *cat* gene was analysed by *cat* assays. Standard deviations calculated for each set of triplicate experiments are indicated.

No difference in the levels of *cat* activity was noted between the constructs containing the C11 or B10 5'-flanking fragments in the sense or antisense orientation. This suggests that the results obtained with the heterologous c-fos and viral tk promoters reflect the presence of positive and negative regulatory elements in the 5'-flanking region of the serine protease genes C11 and B10.

CHAPTER IV

TWO CYTOTOXIC T CELL-SPECIFIC SERINE PROTEASE GENES HAVE DISTINCT SODIUM BUTYRATE-INDUCIBLE ELEMENTS WITHIN THEIR UPSTREAM FLANKING REGIONS

A. INTRODUCTION

Cytolytic lymphocytes play an important role in defense against viral and neoplastic diseases. It now appears that there is more than one way in which these cells can lyse their targets (Berke, 1989, Ostergaard and Clark, 1989). One mechanism involving the directed exocytosis of potential effector molecules from cytoplasmic granules has received considerable attention recently (Young, 1989, Tschopp and Nabholz, 1990). These subcellular organelles contain a family of serine esterases (Bleackley et al., 1988, Jenne and Tschopp, 1988, Haddad et al., 1991), a pore-forming protein called perforin or cytolysin (Podack et al., 1985, Lowrey et al., 1989), proteoglycans (Schmidt et al., 1985, Young et al., 1987, Tschopp and Corzelmann, 1986), a lipase (Grusby et al., 1990) and other less well characterized molecules (Tschopp and Nabholz, 1990). The contents of the granules are released upon interaction with the target and are believed to be active participants in the annihilation of this cell (reviewed in Young, 1989, Tschopp and Nabholz, 1990).

During T cell stimulation, the genes that encode the granule-proteins are transcriptionally activated. Thus, in order to understand how T cell activation is controlled one must identify the elements that regulate the expression of this family of genes. Although the detailed events that control the expression of the individual genes are not known, two of the serine protease genes, C11 and B10 (encoding cytotoxic cell proteases [CCP] 1 and 2, also known as granzymes B and C) were recently shown to be differently regulated (Frégeau and Bleackley, 1991a). Both C11 and B10 contain distinct regulatory elements responsible for their cell-specific distribution and temporal expression. Early observations, made following transfection of cytotoxic T lymphocytes (CTLs) in the presence of sodium butyrate, indicated that C11 and B10 had a different sensitivity to this inducing agent (Frégeau and Bleackley, 1991b). While both 5'-flanking regions were equally effective in driving *cat* transcription in the absence of butyrate, C11 clearly showed a stronger ability to do so in the presence of the agent.

Sodium butyrate has been the subject of many previous studies particularly as an inducer of differentiation. It has been shown to affect gene expression at a number of different levels including chromatin structure (Kruh, 1982, Birren et al., 1987, Birren and Herschman, 1986, Perry and Annunziato, 1989, Oliva et al., 1990), transcription (Mickley et al., 1989, Naranjo et al., 1990, Lazar, 1990, Rius et al., 1990, Chou et al., 1990), and mRNA half-life (Tang et al., 1990). However, there are only a few reports (Gorman and Howard, 1983, Bohan et al., 1989, Tang and Taylor, 1990, Dorner et al., 1989) on the presence of DNA sequences that control butyrate-sensitivity of proximal genes. Here we describe the effect of sodium butyrate on both C11 and B10 endogenous and C11- and B10-directed *cat* gene expression. The reason behind the differential butyrate-responsiveness of the two serine protease genes appears to be defined by distinct genetic elements within the 5'-flanking regions of both B10 and C11. Furthermore, these butyrate-sensitive sequences can confer butyrate-responsiveness to heterologous viral and cellular promoters such as tk and c-fos that are not normally responsive to this agent.

B. MATERIALS AND METHODS

1. *Cell lines and tissue culture*

The cytotoxic T cell line MTL 2.8.2 was generated from CBA/Balb/c mice as described previously (Bleackley et al., 1982). It is an IL2 dependent cell line that can proliferate in the absence of antigen. L is a standard mouse fibroblastic cell line. These cells were cultured in RPMI 1640 medium (Gibco Laboratories, Life Technologies, Inc., Grand Island, NY) supplemented with 10% (v/v) fetal calf serum (HyClone Laboratories, Inc., Logan, Utah) and 100 μM of β-mercaptoethanol. This is referred to as RHFM medium. MTL 2.8.2 cells were maintained in RHFM containing 30 units/ml of recombinant IL2.

2. *Plasmid construction*

The various constructs used in this study have been described in detail elsewhere (Frégeau and Bleackley, 1991a). The parental vectors pGEM1cat and pGEM2cat referred to as pGEMcat are derived from the promoterless vectors pGEM1 and pGEM2 (Promega Corporation). They allow insertion of 5'-flanking fragments at various positions within the multiple cloning site (MCS) region. pFCΔ56 also referred to as pFoscat in this paper was provided by W. Leonard (NIH) and contains the mouse c-fos essential promoter sequences, defined by Gilman et al. (1986) (namely the TATA box without the GC boxes and the cAMP consensus sequences), linked to the cat gene and downstream SV40-derived sequences from pSV0cat in pUC13. pTKcat was provided by R. Miksicek (Heidelberg) and includes the herpes virus thymidine kinase promoter region -109 to +51 (McKnight and Kingsbury, 1982, Graves et al., 1986) ligated upstream of the cat cartridge and polyadenylation site of pSV2cat in pUC8. This promoter region contains the TATA box, the CCAAT box and two SP1 sites (GC boxes).

The deletion fragments were generated from parental plasmids containing the 5'-upstream region of both B10 and C11 (Lobe et al., 1989), respectively 1648 bp (-1617 to +31) and 1078 bp (-961 to +117) excised from genomic clones with EcoR1-Acc1 or EcoR1-Tth1III and subcloned in pUC13. The various fragments shown in this paper were prepared from the parental B10 and C11 plasmids by restriction enzyme cleavage. Table IV.1 presents the various constructs used in the current study with the corresponding portion of B10 or C11 5'-flanking region contained within each vector. A map of B10 and

	Constructs	restricted fragments utilised from the 5'-flanking region	genomic position of the 5'-fragments
B10:	p1648cat	EcoRI-AccI	-1617 to +31
	p1080cat	PstI-AccI	-1049 to +31
	p800cat	HincII-AccI	-769 to +31
	p310cat	BglII-AccI	-279 to +31
	p220cat	HaeIII-AccI	-189 to +31
	p90cat	BglII-HaeIII	-279 to -189
	p336cat	PstI-AluI	-1049 to -713
C11:	p896cat	TaqI-AvaII	-828 to +68
	p495cat	HaeIII-AvaII	-427 to +68
	p311cat	AluI-AvaII	-243 to +68
	p180cat	HinfI-AvaII	-112 to +68
	p439cat	AluI-AluI	-682 to -243
	p184cat	HaeIII-AluI	-427 to -243
	p437cat	HaeIII-HaeIII	-864 to -427
	p220cat	AluI-AluI	-902 to -682
	p255cat	AluI-HaeIII	-682 to -427

TABLE IV.1 Summary of the C11 and B10 5'-end-containing plasmids. Full length C11 and B10 5'-upstream regions were cleaved with the specific restriction enzymes indicated and the various fragments cloned into three distinct *cat* plasmids: pGEMcat, pFCΔ56 and pTKcat after polishing the ends with Klenow and/or T4 DNA polymerase and the addition of HindIII linkers as detailed elsewhere (Frégeau and Bleackley, 1991a). The position of the fragments denoted is in reference to the transcriptional start site +1.

C11 5'-upstream regions is also presented in Figure IV.1.

Plasmid stocks were propagated in *E.coli* strain DH5α and isolated using the alkaline lysis procedure (Birnboim and Doly, 1979). We routinely obtained preparations consisting of more than 90% form 1 (supercoiled) DNA molecules which ensured both consistency and optimal levels of gene expression in transient assays.

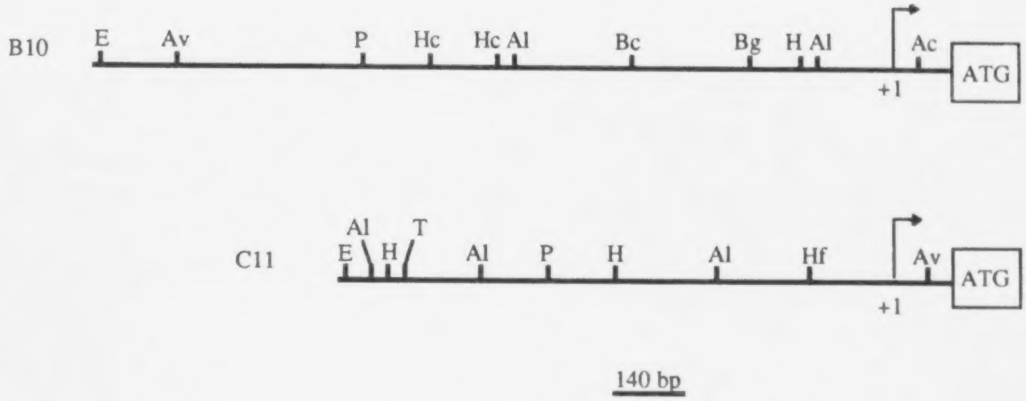

B10
E
Av
P
Hc
Hc Al
Bc
Bg
H Al
Ac
+1
ATG
C11
Al
T
E
H
Al
P
H
Al
Hf
Av
+1
ATG
140 bp

FIGURE IV.1 The B10 and C11 5'-flanking regions showing the restriction endonuclease sites used to generate the various constructs presented. E: EcoRI, Av: AvaII, P: Pst1, Al: AluI, Hc: HincII, Bg: BglII, H: HaeIII, Ac: Acc1, T: Taq1, Hf: HinfI. The transcriptional initiation site is represented by +1. The translational start site ATG is also shown.

3. *Transfections and cat assays*

MTL 2.8.2 cells were transfected following a DEAE dextran protocol adapted and optimized for cytotoxic T cell lines (Frégeau and Bleackley, 1991b). Sodium butyrate was added to a final concentration of 7.5 mM for 13 hours immediately following transfection. Cells were harvested and *cat* assays were performed as previously described (Gorman et al., 1982, Frégeau and Bleackley, 1991b). The concentration in the lysates was determined by the Bio-Rad protein assay. An equal amount of protein (usually 100 µg) from each sample was incubated with 0.014 µCi of 45 mCi/mmol, 0.1 mCi/ml [^{14}C]chloramphenicol (Dupont/NEN products), 4 mM acetyl coenzyme A (Pharmacia) and 0.5 M Tris-HCl pH 7.8 in a final volume of 100-150 µl at 37°C for 4 hours. Reaction products were visualized by autoradiography at room temperature and the relative increases in *cat* activity were determined by liquid scintillation quantification of the acetylated and unacetylated [^{14}C]chloramphenicol after thin layer chromatography. Each series of *cat* assays were performed a minimum of three times and within each set of experiments the parent vectors (pGEMcat, pFCΔ56 and pTKcat) were done in triplicate.

4. *Preparation of RNA and Northern blots*

RNA was extracted using guanidium thiocyanate solubilization and centrifugation over a cesium chloride gradient (Chirgwin et al., 1979). Northern blots for hybridizations were prepared as follows. Total RNA (10-20 µg) was denatured in three volumes of FFMOPS (50% formamide, 6.5% formaldehyde, 1X MOPS ph 7.0) at 65°C for 15 minutes and size-fractionated on a 1% agarose gel containing 0.7% formaldehyde. RNA was transferred onto nylon membrane (Hybond-N, Amersham Ltd.) as described by Thomas (1980). The membranes were cross-linked using the UV Stratalinker^{TM} 1800 set at 1200 µJoulesX100 for 40 seconds then prehybridized for 6-12 hours at 42°C in 50% formamide, 5X SSC (1X SSC: 15 mM sodium citrate, 150 mM NaCl, pH 7.0), 5X Denhardt's solution, 0.1% SDS, 50 mM phosphate buffer pH 6.5, 1 mM NaPyroPO$_4$, 100 mM ATP, 2.5 mM EDTA). Hybridization was carried out in the same buffer with a [^{32}P]nick-translated plasmid of specific activity 1-2 x 10^8 cpm/μg (Bethesda Research Laboratories kit) at 5-10 x 10^6 cpm/ml. Blots were washed at high stringency i.e. 0.1X SSC/0.1% SDS at 56°C for one hour following washes at 42°C with 1X SSC/0.1% SDS and 0.2X SSC/0.1% SDS. Densitometric scanning of autoradiograms was used to quantitate and normalize the amount of RNA in each lane.

5. *Dot blot analysis*

Low molecular weight DNA was recovered from transfected MTL2.8.2 cells following the Hirt procedure (Hirt, 1967). Dot blot analysis was carried out on DNA from Hirt extracts fixed to nylon membranes (Hybond-N, Amersham Ltd.) by filtration through a multiwell minifold (Schleicher & Schuell, Inc.) as recommended by Amersham Ltd. (Technical Bulletin P1/162/85/1). Hybridization of the blots was performed at 42°C under stringent conditions (see above conditions for Northern blots) to [^{32}P]nick-translated pC11 896cat plasmid (1 x 10^8 cpm/μg). Membranes were washed under high stringency in 0.1X SSC/0.1% SDS at 56°C and exposed to Kodak XAR-5 film for one day at room temperature.

C. RESULTS

1. *Effect of sodium butyrate on transfected C11 and B10-driven cat gene expression in MTL2.8.2 cells*

MTL2.8.2 cells were transfected with either pC11 896cat or pB10 1080cat, treated with sodium butyrate for 13 hours at a concentration of 7.5 mM and left to recover for various lengths of time (0, 6, 24 or 48 hours) in butyrate-free medium. Cells were then harvested for *cat* determination. As shown in Figure IV.2 (panel A), the C11-driven *cat* gene activity in MTL2.8.2 cells treated with butyrate for 13 hours was higher (2 fold) than that in untreated cells. Following discontinuation of the drug treatment, the level of *cat* activity increased 3 fold (6 fold above the untreated sample) and remained high up to 48

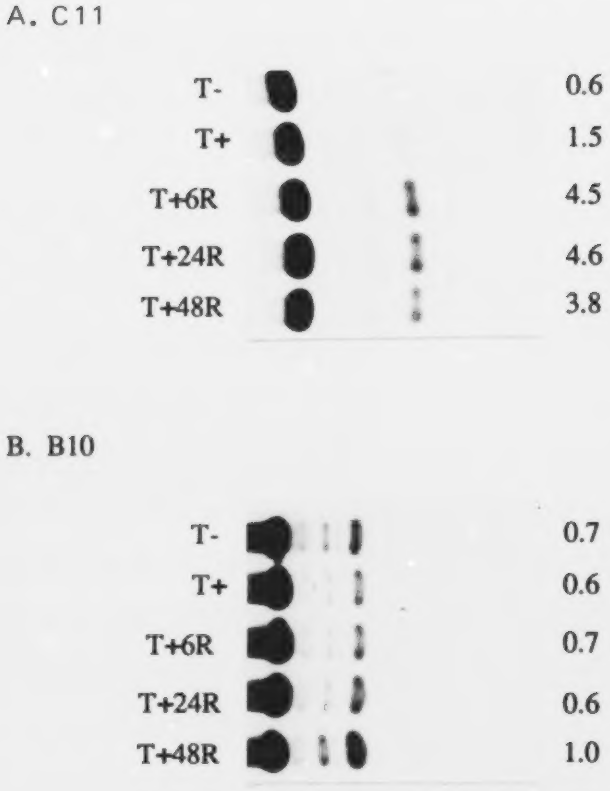


FIGURE IV.2 Effect of sodium butyrate on C11- and B10-directed *cat* activity. MTL2.8.2 cells were transfected with either pC11 896cat (panel A) or pB10 1080cat (panel B) and treated with sodium butyrate for 13 hours at a concentration of 7.5 mM. Following this step, the cells were either collected immediately or allowed to recover for 6, 24, or 48 hours at 37°C/5% CO_2 atmosphere in butyrate-free medium. Cells were then harvested for *cat* determination (see Materials and Methods). The absolute percentage of chloramphenicol acetylation is given on the right. T(-) refers to untreated transfected MTL2.8.2 cells, T(+) to butyrate-treated transfected cells and R to recovery time. Exposure time was 24 hrs at room temperature for C11 and 15 days at room temperature for B10.

hours in the recovery medium. B10 (panel B), on the other hand, showed no modulation in the *cat* activity levels following the addition of butyrate and after its removal from the medium. In order to determine that this difference between C11 and B10 was not due to a different number of plasmid copies introduced into MTL2.8.2 cells, Hirt extractions were performed as described in Materials and Methods. In both cases, the same number of DNA molecules was found (see Figure IV.3).

2. *Effect of sodium butyrate on the level of endogenous C11 and B10 gene expression in MTL2.8.2 cytotoxic T cells*

MTL2.8.2 cells at a density of 1.5 x 10^5 cells/ml (two days following their passage in IL2 (30 units/ml) were exposed to sodium butyrate for 13 hours at a concentration of 7.5 mM. The cells were then washed and left at 37°C in a 5% CO_2 incubator in drug-free medium for 0, 6, 24 or 48 hours before harvest for RNA extraction. Northern blots were prepared and successively probed with cDNA for B10, C11 and γ-actin. The steady-state level of γ-actin was not changed following butyrate treatment and was therefore used to normalize the amount of RNA in each lane.

Figure IV.4 shows the densitometry plots obtained from analysis of the Northern blots probed with C11 (panel A) and B10 (panel B). Each diagram represents the steady-state levels of mRNA corresponding to the endogenous C11 and B10 genes isolated from untransfected MTL2.8.2 cells immediately following sodium butyrate treatment and various times after its removal. A two fold increase in the level of mRNA for C11 was detected 13 hours following the addition of sodium butyrate (compare columns 1 and 2, panel A). This increase was not reversible after discontinuation of the treatment. Indeed, a further two fold enhancement (i.e. 4 fold above the untreated cells) was measured after 24 hours in the butyrate-free medium (compare columns 1 and 4). The steady-state level of C11 mRNA remained high up to 48 hours post-treatment. A different profile of expression was observed for B10 where no significant changes in the steady-state level of its mRNA were detected immediately after the 13 hour-butyrate treatment or following the recovery periods

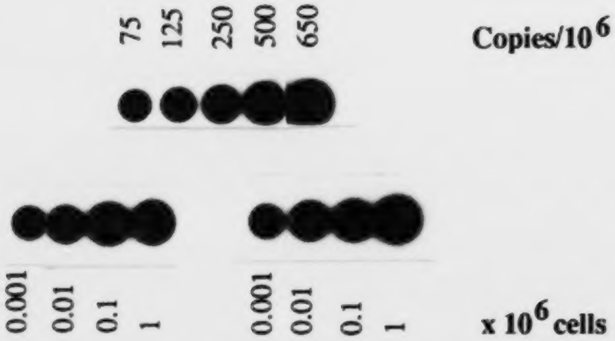

75
125
250
500
650
Copies/10⁶ cells
0.001
0.01
0.1
1
0.001
0.01
0.1
1
x 10⁶ cells

FIGURE IV.3 Determination of the number of copies of pC11 896cat and pB10 1080cat plasmids introduced in MTL2.8.2 cells by transfection. MTL2.8.2 cells were transfected with 15 µg of pC11 896cat and pB10 1080cat using the DEAE dextran protocol outlined in Materials and Methods. At harvest, cells were washed twice with PBS and low molecular weight DNA was extracted from these cell pellets as detailed by Hirt (Hirt, 1967). Standards for the number of copies were prepared using pC11 896cat and an equivalent of 1 copy/10^6 cells was set as the base for the calculations. Each dot represents the equivalent of 10^6 cells.

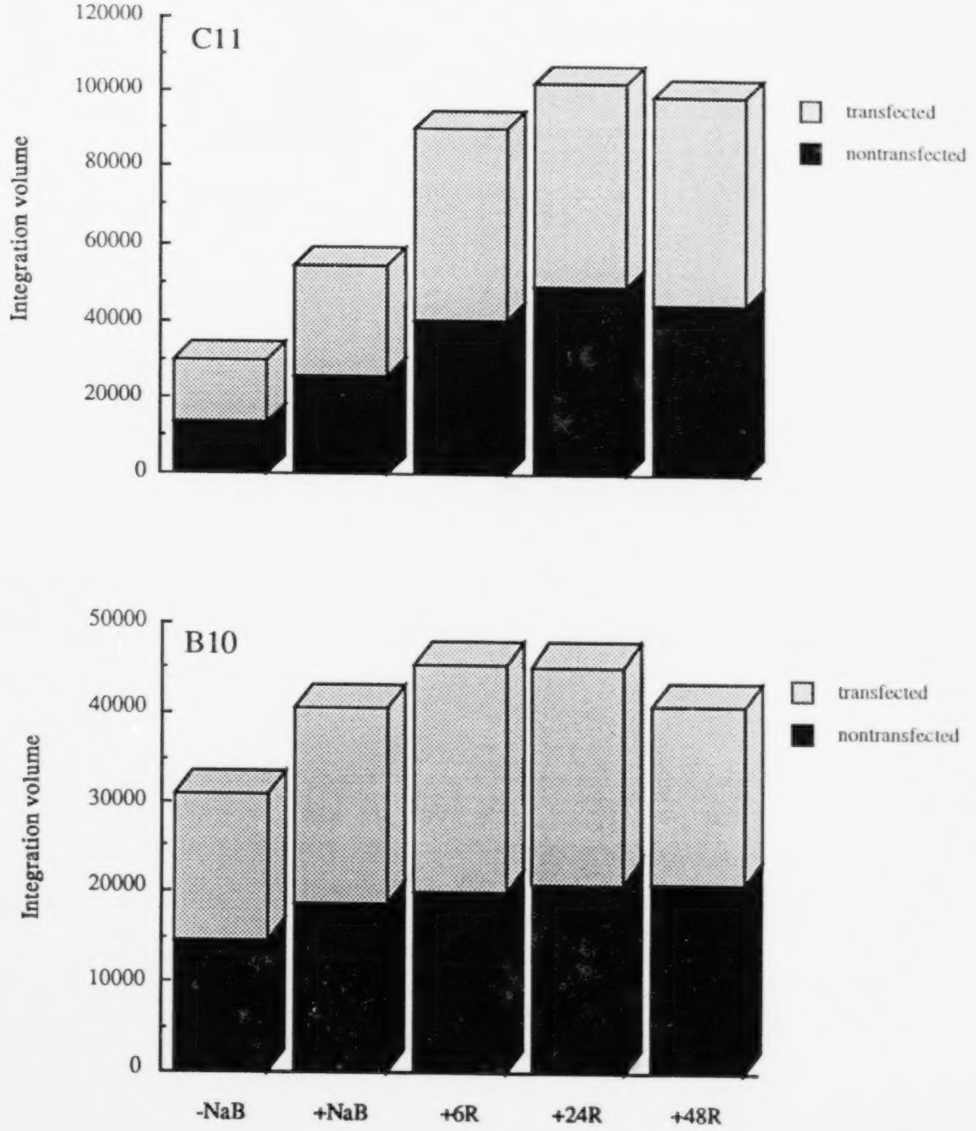


FIGURE IV.4 Densitometry plots of the steady-state level of endogenous mRNA for C11 and B10 serine protease genes following sodium butyrate treatment. MTL2.8.2 cells were transfected with constructs pC11 896cat and pB10 1080cat or used nontransfected and were exposed to butyrate at a concentration of 7.5 mM for 13 hours. Cells were allowed to recover in inducer-free medium for 0, 6, 24, or 48 hours before harvest for RNA extraction. Total RNA (20 µg) was denatured and size-fractionated on a 1% agarose/0.7% formaldehyde gel. Blots were prepared and probed successively with cDNAs for C11, B10 and γ-actin. The autoradiograms were quantitated on a densitometry scanner. The amount of RNA in each lane was normalized using the γ-actin signal. Column 1: untreated MTL2.8.2 cells, column 2: butyrate-treated cells examined immediately after the 13 hour-treatment, column 3: butyrate-treated cells following 6 hours recovery in butyrate-free medium, column 4: following 24 hours recovery, and column 5: following 48 hours recovery.

in the drug-free medium (panel B).

In order to establish that the effect of sodium butyrate on C11 and B10 endogenous gene expression was not altered by transfection manipulations, MTL2.8.2 cells (at a density of 1.5 x 10^5 cells/ml) were transfected with either pC11 896cat or pB10 1080cat plasmids as described in Materials and Methods prior to their treatment with the agent. As above, the length of treatment was set for 13 hours at 7.5 mM and the recovery periods were 0, 6, 24 or 48 hours in drug-free medium. Following RNA extraction, Northern blots were prepared and probed with the same series of cDNA as was used for the untransfected samples.

The densitometry plots obtained for this series of samples are also shown in Figure IV.4 where panel A represents blots hybridized with C11 and panel B with B10. Both transfected and untransfected samples gave a similar profile of expression after the addition of butyrate and following its removal.

3. *The 5'-flanking regions of C11 and B10 can confer butyrate-responsiveness to heterologous promoters*

To determine if butyrate-sensitivity of the serine protease 5'-upstream regions was dependent on sequences found within the promoters, the large 5'-fragments of C11 and B10 were fused to heterologous sequences i.e. the c-fos promoter in pFoscat and the viral herpes tk promoter in pTkcat vectors. Following transfection into MTL2.8.2 cells and butyrate treatment (7.5 mM for 13 hours), cells were supplemented with fresh medium depleted of inducing agent and incubated for a further 35 hours before harvest. The data presented in Table IV.2 demonstrates that although the parental vectors (pFoscat and pTkcat) were not significantly sensitive to butyrate, the fusion of the C11 and B10 5'-flanking regions conferred upon them butyrate-responsiveness. The effect was particularly notable with the c-fos basal promoter where a 11 and 3.1 fold induction in *cat* activity was

Constructs	-Butyrate		+Butyrate		Fold Stimulation	
pGEMcat	1.0	(0.27)	1.0	(0.30)	1.0	(1.1)
pB10 1080cat	9.0	(2.4)	10.0	(3.1)	1.2	(1.3)
pC11 896cat	10.0	(2.7)	55.0	(17.0)	5.5	(6.3)
pFoscat	1.0	(0.88)	1.0	(1.2)	1.0	(1.4)
pB10F 1080cat	4.9	(4.7)	15.0	(18.0)	3.1	(3.8)
pC11F 896cat	3.9	(3.4)	41.0	(49.0)	11.0	(12.0)
pTKcat	1.0	(5.3)	1.0	(7.4)	1.0	(1.4)
pB10Tk 1080cat	1.8	(9.8)	3.7	(28.0)	2.1	(2.8)
pC11Tk 896cat	1.2	(6.5)	2.1	(16.0)	1.8	(2.4)

TABLE IV.2 Differential butyrate-sensitivity of the 5'-flanking regions of C11 and B10 when placed in front of different promoters. MTL2.8.2 cytotoxic T cells were transfected with 15 µg of plasmid DNA following a DEAE dextran procedure as described elsewhere (Frégeau and Bleackley, 1991b). One series was treated with sodium butyrate for 13 hours at a concentration of 7.5 mM then fed fresh medium without drug for another 35 hours before harvest and *cat* determination. Values given are relative to the parental vector set at 1.0 and represent the mean of three or more experiments which agreed within 10%. Within each series of experiments, the parental vectors were done in triplicate. Numbers in parentheses represent absolute values of *cat* activity (% chloramphenicol acetylation).

measured for C11 and B10 respectively. In its natural context and when fused to the c-fos promoter, C11 was better at conferring butyrate-sensitivity than was the B10 5'-end (5.5 fold induction (C11) versus 1.2 (B10) in natural context). However, when fused to the tk promoter, both C11 and B10 were roughly equal at stimulating butyrate-sensitivity (1.8 (C11) and 2.1 (B10)). Thus, it appears that the addition of approximately 1 kilobase sequence from B10 or C11 is sufficient to increase stimulation by butyrate in a number of different promoter contexts.

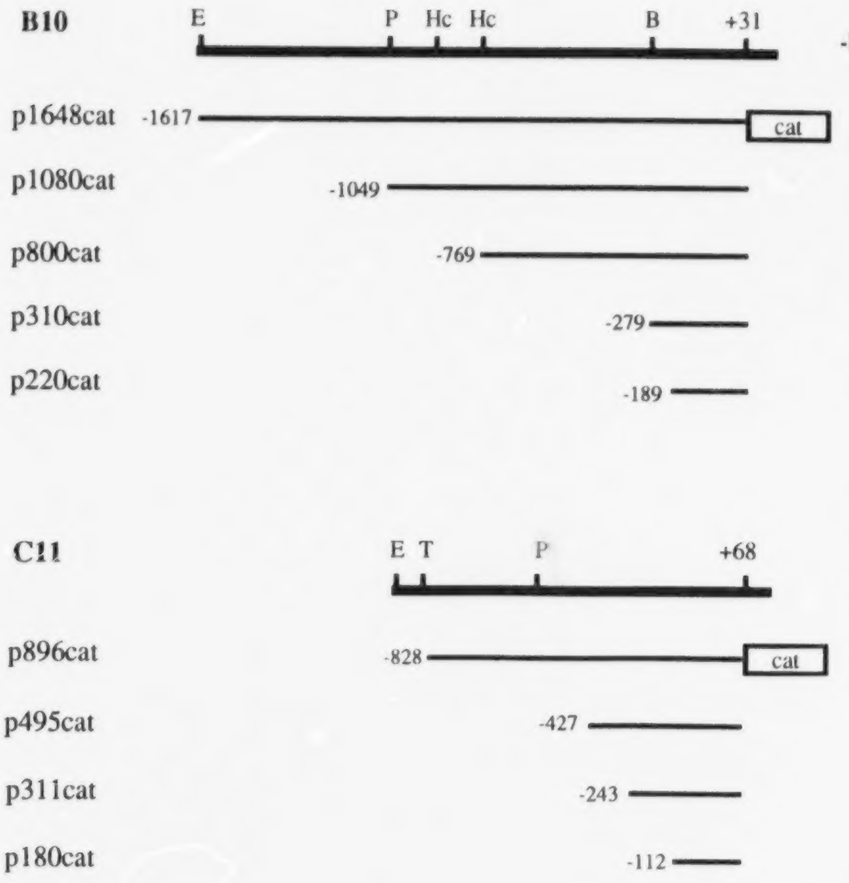
4. *Mapping butyrate-responsive sequences within C11 and B10 5'-flanking regions*

We next examined the 5'-flanking regions of the two serine protease genes to define the genetic elements that could be responsible for the butyrate sensitivity observed. A series of deletion fragments spanning the 5'-ends of both C11 and B10 genes was generated using restriction cleavage sites. These fragments were fused to the *cat* reporter gene in pGEMcat and transfected into MTL2.8.2 cells. Following butyrate treatment (7.5 mM for 13 hours), cells were supplemented with fresh medium free of inducer and incubated for a further 35 hours before harvest. Figure IV.5 represents a detailed dissection of the 5'-end of B10 (panel A) and C11 (panel B). In its natural context, B10 was shown to be nonresponsive to butyrate (see Table IV.2) and the lack of butyrate-sensitive regions confirms this observation. C11 was quite different in that at least one butyrate-inducible region was identified. The deletion of the sequences between -828 and -243 did not lead to a significant decrease (22%) in the fold stimulation by butyrate. However, removal of the sequences downstream from -243 (i.e. -243 to -112) resulted in a 3.6 fold (72%) loss in butyrate-inducibility. This denoted a proximal site responsive to sodium butyrate induction.

5. *C11 and B10 5'-flanking regions have different butyrate sensitivity when placed within heterologous promoter contexts*

MTL2.8.2 cells were transfected with the pFCΔ56 series of constructs and following treatment with butyrate for 13 hours, were left in drug-free medium for 35 hours before *cat* determination. Figure IV.6 indicates that for B10, the deletion of the sequences between -1617 and -1049 led to an increase in the fold stimulation indicating the presence of sequences that prevented induction by butyrate within these boundaries. Deletion of B10 sequences downstream from -1049 to -769 resulted in a 2.6 fold loss (61%) in butyrate-inducibility and identified a distal site responsive to butyrate induction (-1049 to -769) which was confirmed by construct p336Fcat. This site was not revealed in the B10

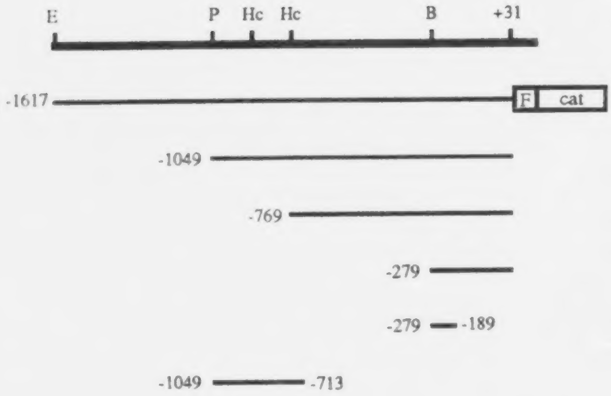
FIGURE IV.5 Effect of sodium butyrate on B10 and C11/cat fusion constructs in MTL2.8.2 cytotoxic T cells. Fragments of the 5'-flanking sequences of B10 (upper panel) and C11 (lower panel) were ligated upstream of the *cat* gene in pGEMcat vectors as described under Materials and Methods and were transfected into MTL2.8.2 cells. Cells were subjected to butyrate at a concentration of 7.5 mM for 13 hours. Following a media change, cells were allowed to recover for 35 hours before harvest and *cat* determination. The fold stimulation was determined by assaying both untreated and treated cells. Numbers presented are *cat* activities relative to the parent promoterless pGEMcat plasmid. Absolute values of chloramphenicol acetylation normalized to 1.0 were for untreated cells 0.27 and for butyrate-treated cells 0.30. Each value represents the mean of at least three experiments. Abbreviations for the restriction enzyme cleavage sites are E: EcoRI, P: Pst1, Hc: HincII, B: BglII, T: Taq1.


B10
E
P
Hc
Hc
B
+31
-1617
cat
-1049
-769
-279
-189
C11
E
T
P
+68
-828
cat
-427
-243
-112

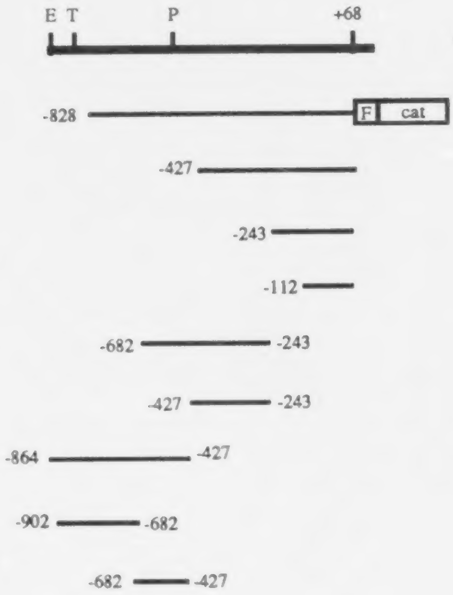
B10	-Butyrate	+Butyrate	Fold stimulation
p1648cat	4.0	3.8	1.0
p1080cat	9.0	10	1.1
p800cat	7.5	10	1.3
p310cat	6.4	6.0	0.9
p220cat	2.4	2.1	0.9

C11	-Butyrate	+Butyrate	Fold stimulation
p896cat	10	55	5.5
p495cat	6.5	28	4.3
p311cat	12	51	4.3
p180cat	1.7	2.0	1.2

FIGURE IV.6 Butyrate-responsiveness of the c-fos promoter conferred by B10 and C11 5'-upstream sequences. Fragments of the 5'-flanking regions of B10 (upper panel) and C11 (lower panel) genes were ligated upstream of the minimal fos promoter in pFCΔ56 and transfected into MTL2.8.2 cells. Following butyrate exposure for 13 hours at a concentration of 7.5 mM, cells were resuspended in fresh medium and incubated for a further 35 hours before harvest and *cat* determination. The *cat* activities relative to the parental pFCΔ56 plasmid are shown. The absolute values for pFCΔ56-driven *cat* activities were 0.88% for untreated cells and 1.2% for butyrate-treated cells. The fold induction was determined by comparing untreated and treated samples. All values given represent the average of at least three experiments. For abbreviations see Figure IV.5.


E
P
Hc
Hc
B
+31
-1617
F
cat
-1049
-769
-279
-279
-189
-1049
-713

B10	-Butyrate	+Butyrate	Fold stimulation
p1648Foscat	2.1	2.0	1.0
p1080Fcat	4.9	15	3.1
p800Fcat	4.1	4.3	1.0
p310Fcat	5.7	5.8	1.0
p90Fcat	4.0	4.2	1.0
p336Fcat	0.5	1.3	2.6


E
T
P
+68
-828
F
cat
-427
-243
-112
-682
-243
-427
-243
-864
-427
-902
-682
-682
-427

C11	-Butyrate	+Butyrate	Fold stimulation
p896Foscat	3.9	41	11
p495Fcat	0.6	1.9	3.2
p311Fcat	1.9	14	7.5
p180Fcat	0.6	0.6	1.0
p439Fcat	13	36	2.8
p184Fcat	0.8	0.7	0.9
p437Fcat	1.7	3.6	2.1
p220Fcat	0.6	0.6	1.0
p255Fcat	0.5	1.3	2.5

natural promoter context. A further deletion of sequences between -769 to -279 resulted in an insignificant decrease in stimulation by butyrate (17%).

For C11, the region between -828 to -427 appears to contain sequences that respond to butyrate since its deletion resulted in a 71% loss in fold stimulation. In the natural promoter context, the removal of this region showed a minor effect in sodium butyrate inducibility. Further deletion of sequences upstream from -243 (-427 to -243) resulted in an increase (2.4 fold or 57%) in butyrate-inducibility. The region between -243 to -112 defined as butyrate-sensitive in the C11 natural context (Figure IV.5) behaved similarly when placed upstream of the c-fos promoter i.e. a 7.5 fold (87%) decrease in fold stimulation was measured when it was deleted. Constructs p439Fcat, p437Fcat and p255Fcat all contain sequences between -682 to -427 and all showed a stimulation of *cat* activity (2 to 3 fold) after butyrate treatment. Constructs p184Fcat and p220Fcat further indicated that if this region was removed, butyrate-inducibility was also lost. This series of deletion constructs delineated a distal butyrate-inducible area in C11 (-682 to -427) indicating the presence of butyrate-sensitive sequences between these boundaries.

D. DISCUSSION

Many reports have indicated that sodium butyrate can modulate gene activity in a variety of cell lines. It has also been shown that this agent can have different consequences on the induction of specific RNAs in the same cell (see Table I.4 -Introduction of the thesis). We demonstrated, in an earlier study, that sodium butyrate treatment induces C11-driven *cat* gene expression five fold over that of B10-driven *cat* gene expression in the cytotoxic T cell line MTL2.8.2 (Frégeau and Bleackley, 1991b). In this report, we investigated the reason behind this differential sensitivity to butyrate.

The length of the sodium butyrate treatment and the concentration of inducer chosen for our experiments were based on a previous optimization study for transient expression in

MTL2.8.2 cells (Frégeau and Bleackley, 1991b). Many other groups have used concentrations ranging from 1 to 10 mM for 30 minutes to 7 days and measured changes in mRNA level from 5 to 80 fold depending on the experimental conditions (see Table I.4 - Introduction of the thesis). Undoubtedly, shorter or longer exposures will have different consequences on the level of modulation of gene expression by butyrate. In the current report, the transfected *cat* gene expression driven by the C11 5'-flanking region was induced two fold following exposure to butyrate (7.5 mM) for 13 hours and was further increased to four fold after 6 hours in butyrate-free medium (Figure IV.2, panel A). The *cat* activity remained high up to 48 hours in this recovery buffer. For the B10-directed *cat* gene expression, the addition or discontinuation of butyrate had no effect as the levels of *cat* activity measured were identical to those of untreated cells (panel B).

Comparing Figures IV.2 and IV.3 revealed a similar pattern of expression for the endogenous genes. C11 endogenous expression was induced approximately two fold when analysed immediately after the 13 hour treatment (Figure IV.3, panel A; compare columns 1 and 2). On the other hand, B10 endogenous mRNA level showed basically no change (panel B). Another interesting observation was that following discontinuation of the treatment i.e 6 hours or 24 hours after removal of the drug, the steady-state levels of C11 mRNA increased even more reaching five fold (compare columns 1 and 4). This increase was persistent up to 48 hours post-treatment where the last sample was collected. This first series of experiments provided evidence that the differential sensitivity to butyrate observed between C11 and B10 was reflected at the level of endogenous as well as transfected gene expression. Previous investigators indicated that the pattern of modulation following removal of sodium butyrate is dependent on the gene analysed (Mickley et al., 1989). Their conclusion was reached after the examination of unrelated genes such as the multidrug resistance gene encoding the mdr-1/P-glycoprotein and the transforming growth factor α. Our results indicate that this statement can even be extended to members of the same gene family such as C11 and B10.

The experiments described above also suggested the possibility that specific sequences or areas within the 5'-upstream region of C11 could be particularly sensitive to butyrate. Table IV.2 showed that it is indeed the case. When the 5'-flanking regions of C11 and B10 were fused to heterologous promoters such as the cellular fos and viral tk, both were able to confer butyrate responsiveness to the normally insensitive promoters. C11 showed sensitivity to the inducer in its natural context (see Figure IV.2, panel A) and it was therefore not surprising to see that it could confer sensitivity to other genes. However, B10 showed no sensitivity to butyrate in its natural environment (Figure IV.2, panel B) and yet was able to confer responsiveness to heterologous promoters. The dissection of the 5'-flanking region of B10 provided an explanation for this interesting observation.

As presented in Figures IV.5 and IV.6, distinct butyrate-sensitive regions were identified for the C11 and B10 genes. In the case of C11, sequences between -828 and -427 and between -243 and -112 were defined as responsive in the natural C11 promoter context. Although the level of responsiveness was low for the area delineated by nucleotides -828 to -427, its butyrate sensitivity was confirmed and further narrowed to nucleotides -682 to -427 when fused to the c-fos basal promoter where a 3.5 fold increase in *cat* activity was measured (compare Figures IV.5 and IV.6). Sequences between -243 and -112 had the most impact on butyrate stimulation in both promoter contexts with a 7.5 fold increase upon fusion to c-fos. These results are consistent with those of Gorman and Howard (1983) who observed a 2 to 10 fold enhancement in SV40-directed *cat* activity in cells that had been treated for 12 hours with 10 mM sodium butyrate. They speculated that one of the possible mechanisms by which butyrate might facilitate the transient expression of exogenously supplied foreign genes was to promote their assembly into "active" or "open" chromatin. It was also suggested that possible targets of the action of butyrate could be the cellular DNA-binding proteins. These presumably become post-translationally modified following exposure to butyrate (Partington et al., 1984, Naranjo et al., 1990, Stoddart et al., 1989). A direct consequence of this action would be to alter the network of interactions between transcriptional factors that bind to regulatory regions and change the fate of gene expression. It is more likely that constructs containing sequences from -682 to -427 or -243 to -112 are sensitive to butyrate because these sequences are target sites for specific factors which are particularly affected by the modifications induced by the agent. Indeed, the C11 butyrate-sensitive sequences map to DNAse1 hypersensitive regions previously defined (Lobe et al., 1989). Such regions usually correspond to chromatin domains that are transcriptionally active. This suggests that the genetic elements that are contained within the butyrate-responsive areas may attract specific factors which are involved in the activation of the C11 gene.

For B10, sequences from -1049 to -769 were the only ones that showed butyrate sensitivity. In addition, this responsiveness was apparent only when placed upstream of the basal c-fos promoter (compare Figures IV.5 and IV.6, panels A). Confirmation of the sensitivity of this area came from construct p336Fcat where nucleotides from -1049 to -713 showed a 2.6 fold enhancement in *cat* activity over the other deletion constructs. As in C11, these sequences correspond to a DNAse1 hypersensitive region in B10 and may contain genetic elements required for the activation of that gene. This series of experiments indicated that responsiveness to butyrate is dependent on the promoter and the sequences in the vicinity of that promoter.

Similar observations were obtained from groups who studied the effect of sodium butyrate on expression of transfected DNA molecules. Tang and Taylor (1990) demonstrated that a nonresponsive promoter such as that of adenine phosphoribosyltransferase (APRT) could be induced by butyrate by placing an inducible Moloney sarcoma virus (MSV) enhancer-promoter upstream from it. It was suggested that the acetylation of histones by butyrate may change the chromatin structure surrounding the MSV enhancer in a specific way to open up new avenues for transcription factors to bind nearby promoters. Another possibility was the activation of trans-acting factors following modifications induced by butyrate; factors which would bind to the MSV enhancer and participate in the formation of the transcriptional complexes to activate APRT. In the same vain, Dorner et al. (1989) demonstrated that treatment of CHO cells with butyrate increased the expression of erythropoietin, Factor VIII and von Willebrand factor from stably integrated and amplified genes which utilize the adenovirus major late promoter in combination with the SV40 enhancer for transcription. They indicated that the combination of promoter and enhancer is important in determining the responsiveness of integrated transcription units to butyrate. The same conclusion had been reached by Gorman and Howard, who showed that the SV40 promoter was very sensitive to butyrate but that the deletion of the enhancer reduced this induction by a factor of 6 to 10 (Gorman and Howard, 1983).

The results represented in Figures IV.5 and IV.6 are summarized in Figure IV.7. Cis-acting genetic elements within B10 and C11 5'-flanks appear to include the positive regulatory regions -279 to -189 for B10 and -682 to -427 and -243 to -112 for C11 and the negative regulatory regions -1617 to -1049 for B10 and -427 to -243 for C11 (Frégeau and Bleackley, 1991a). The proximal C11 target site for sodium butyrate induction was located between -243 to -112 relative to the transcriptional start site (+1) which correspond to the area comprising the regulatory elements reported to exert a positive effect on C11 expression. Because the boundaries of the C11 promoter have not been defined yet, we do not know if any of the binding sites for RNA polymerase II basal transcription are also part of this proximal site for sodium butyrate induction. Fragment -243 to -112 contains sequences homologous to AP1 (Angel et al., 1987), AP2 (Imagawa et al., 1987), NFκB (Lenardo and Baltimore, 1989) and to SV40 and Moloney murine leukemia virus enhancer core (Boral et al., 1989). The presence of these genetic elements may contribute to butyrate-sensitivity of the region. Indeed, some of them represent recognition blocks for transcription factors whose activity has been shown to be modulated post-translationally. AP1 is the target site for fos and jun which are modified following phosphorylation events

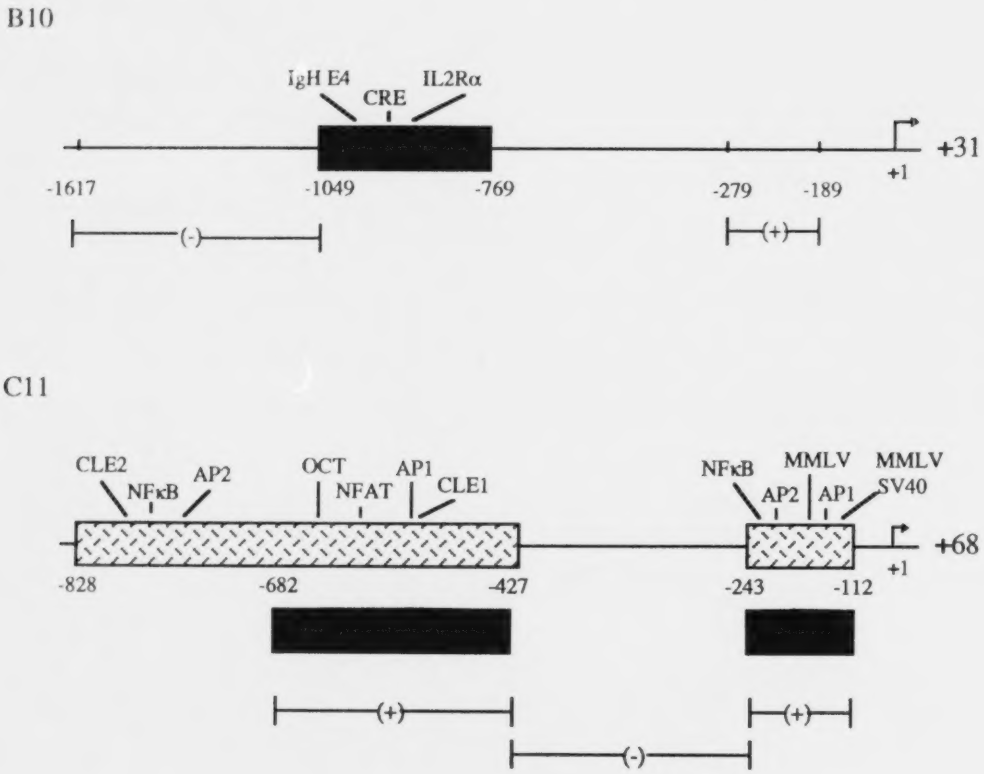


FIGURE IV.7 Summary of the butyrate-responsive elements mapped within the B10 and C11 5'-upstream regions and their relation to the cis-acting regulatory elements defined by Frégeau and Bleackley (1991a). Butyrate-sensitive sequences are represented by boxes; those identified in the natural serine protease C11 5'-context are dotted and those delineated in B10 and C11 with the heterologous promoter c-fos are filled. Negative (-) and positive (+) symbols delineate the regulatory areas distinct to B10 and C11 that down-regulate and up-regulate transcription. Sequences within the butyrate-sensitive regions which are homologous to regulatory motifs identified in numerous genes are also shown. IgH E4, immunoglobulin heavy chain enhancer core E4; CRE, cAMP responsive element; IL2Rα, IL2 receptor alpha negative element; CLE1 or CLE2, conserved lymphokine element 1 or 2; NFκB, nuclear factor of kappa light chain in B cells; AP1 or AP2, activating protein 1 or 2; OCT, octamer motif; NFAT, purine box of the binding site of nuclear factor of activated T cells; MMLV, Moloney murine leukemia virus enhancer core, SV40, simian virus 40 enhancer core. The numbering is in reference to the transcription start site +1.

(Boyle et al., 1991). NFκB sequences binds NFκB which is activated after its release from a complex in which it is bound to inhibitor IκB. Phosphorylation reactions mediate this release (Ghosh and Baltimore, 1990). These sites could also bind other factors which would be sensitive to butyrate's action.

As yet, there are no reports demonstrating unequivocally that sodium butyrate causes changes in the binding or function of any cellular transcription factors. However, acetylation and phosphorylation events of nuclear proteins have been noted following butyrate treatment (Levy-Wilson, 1981; Boffa et al., 1981; Kruh, 1982; Naranjo et al., 1990). Thus, sodium butyrate may alter directly or indirectly the post-translational modifications of C11 transcriptional factors or proteins that act upon these for their activity, and change the fate of gene expression.

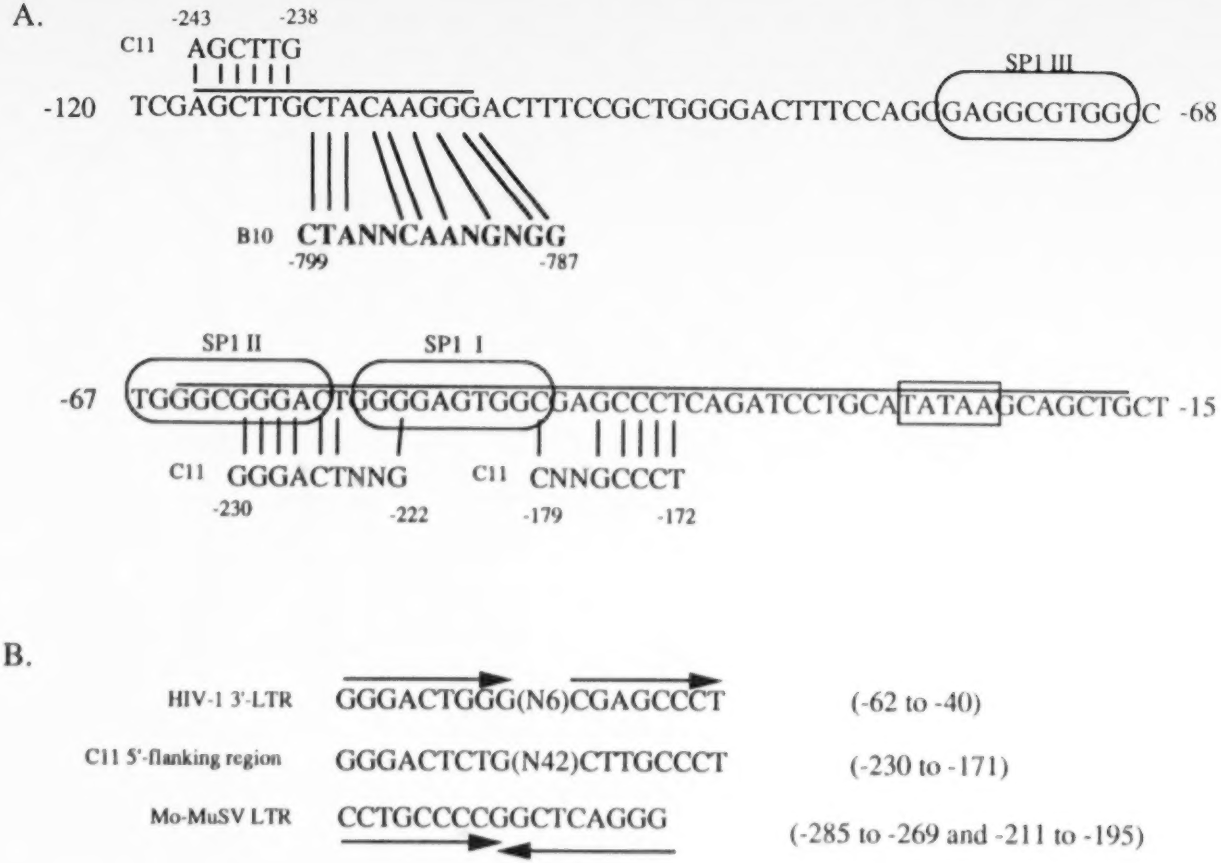
The region of C11 between -682 and -427 contain sequences homologous to AP1, an octamer (OCT) motif (Wirth et al., 1987), a conserved lymphokine element (CLE1) binding site (Arai et al., 1990) and the purine box of the IL2-specific nuclear factor binding site NFAT (Fujita et al., 1986; Randak et al., 1990). This area of the 5'-end of C11 exerted a major effect on butyrate induction when placed in the c-fos promoter context (compare Figures IV.5 and IV.6). A possible explanation for such an effect could be that the C11 DNA-binding factors are sensitive to modifications induced by butyrate and participate in the formation of transcriptional complexes along with other proteins that recognize the basal fos promoter. In this instance, the combination of the C11 and fos transcriptional factors induces the level of *cat* activity and the response to butyrate.

In the case of B10, the known regulatory elements that are located within the butyrate-responsive region (-1049 to -769) correspond to the cAMP responsive element (CRE) (Roesler et al., 1988), an IL2 receptor α chain (IL2Rα)-specific element binding site (Smith and Greene, 1989) and an immunoglobulin heavy chain enhancer core sequences (Lenardo et al., 1987). Similarly to C11, these genetic elements could be particularly sensitive to the effects of butyrate and along with the c-fos proteins, induce the formation of complexes which activate transcription of gene expression. Many reports have correlated phosphorylation status with activity of CREB (Lamph et al., 1990, Gonzalez et al., 1991). The combination of the B10 factors and the c-fos DNA-binding proteins appears to be required in this instance since the fragment -1049 to -769 in the context of the B10 5'-flanking region does not increase *cat* gene expression following butyrate addition. These results emphasize the combinatorial aspect of gene regulation whereby modifications of transcriptional proteins mediated by an inducer such as sodium butyrate will have profound effects on gene activation.

Sodium butyrate has been shown to stimulate HIV-1 gene expression (Bohan et al. 1987). This specific induction was recently attributed to at least two LTR inducible regions, a distal site -117 to -103 and a proximal site -65 to -17 present within the 3'-LTR (Bohan et al. 1989). Figure IV.8 (panel A) shows the homologies between the butyrate-inducible sequences found within HIV-1 3'-LTR and the C11 and B10 5'-flanking regions. It was very interesting to find that the C11 region comprising -243 to -112 sequences gave the highest degree of identity with HIV-1 sequences. This region was butyrate-sensitive in both contexts examined i.e. natural and cellular fos. These areas of homology between these two unrelated genes i.e. AGCTTG (-243 to -238), GGGACTNNG (-230 to -222), CNNGCCCT (-179 to -172) point to novel butyrate-inducible consensus sequences. No homology was detected within the C11 region delineated by nucleotides -682 to -427. The B10 fragment -1049 to -769 also contain sequences that are found in HIV-1 butyrate-sensitive regions at position -799 to -787. In HIV-1, the butyrate-sensitive sequences map to the Sp1 binding site I and II and within the TATA box binding region suggesting the importance of Sp1 and TATA box binding factors in sodium butyrate induction of HIV-1 gene expression.

Tang and Taylor (1990) were able to convert the butyrate-insensitive APRT promoter into a sensitive one by positioning the MSV enhancer in its vicinity. By comparing the Mo-MSV enhancer (Thiesen et al., 1988) with butyrate-sensitive regions

FIGURE IV.8 Homologies between HIV-1 3'-LTR butyrate-responsive sequences and those of C11 and B10 5'-flanking regions. Panel A: the nucleotide sequence of HIV-1 3' LTR (isolate BH10) comprising both butyrate-sensitive regions mapped by Bohan et al. (1989). HIV-1 butyrate-responsive areas are indicated by a line above the sequences (-117 to -103 and -65 to -17). Sequences from C11 and B10 butyrate-responsive regions that show homology to HIV-1 3' LTR elements are given above and underneath with C11 as plain letters and B10 as bold. The numbering refers to the position of the sequences within the 5'-flanking regions of C11 and B10. N refers to pyrimidine or purine. The TATA box and SP1 binding sites I, II and III identified within the HIV-1 3' LTR are also shown. Panel B: the 17 bp homologous sequence found between HIV-1 3' LTR, C11 5'-end butyrate-sensitive areas and Moloney murine sarcoma virus LTR enhancer. The respective position of this 17 oligomer within each context is given.


A.
-243
-238
C11
AGCTTG
SP1 III
-120
TCGAGCTTGCTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGGCC
-68
B10
CTANNCAANGNGG
-799
-787
SP1 II
SP1 I
-67
TGGGCGGGACTGGGAGTGGCGAGCCCTCAGATCCTGCATATAAGCAGCTGCT
-15
C11
GGGACTNNG
C11
CNNGCCCT
-230
-222
-179
-172
B.
HIV-1 3'-LTR
GGGACTGGG(N6)CGAGCCCT
(-62 to -40)
C11 5'-flanking region
GGGACTCTG(N42)CTTGCCCT
(-230 to -171)
Mo-MuSV LTR
CCTGCCCCGGCTCAGGG
(-285 to -269 and -211 to -195)

from the HIV-1 3' LTR and C11, we identified a common 17 bp sequence in all three nucleotide segments (Figure IV.8, panel B). This element appears to be comprised of two parts, a nonamer and an octamer which can be separated by a different number of nucleotides, 6 in the case of HIV-1, 42 in C11 and none in Mo-MuSV. It was interesting to note that in HIV-1 3' LTR and in C11, the nonamer and octamer sequences were found in the same orientation i.e. head to tail while in Mo-MuSV, both elements were facing each other. In addition, in the Mo-MuSV enhancer, the nonamer sequence was found in the reverse orientation i.e. reading 3' to 5' instead of 5' to 3' as in C11 and HIV-1. The presence of this 17 nucleotide sequence, found at two different locations within the Mo-MuSV and once within HIV-1 3' LTR and 5'-end of C11, suggests an important role in butyrate-responsiveness. Further experiments are required to determine the mechanism(s) by which these elements could promote butyrate-stimulation when placed within different genomic contexts.

In summary, we have shown that two members of the serine protease multigene family, namely C11 and B10, have a different level of sensitivity to the inducer sodium butyrate. We found that the increase noted for C11-driven transfected *cat* gene expression is paralleled by a similar enhancement in the endogenous steady-state level of C11 mRNA. As well, the butyrate insensitivity of the B10-driven *cat* gene expression was also apparent at the level of B10 endogenous gene activity where no modulation was observed after exposure to the inducer. We have further demonstrated that this differential stimulation of C11 and B10 is attributed to the presence of distinct responsive sequences located within the 5'-flanking region which confer butyrate responsiveness to heterologous promoters. In the natural promoter context, the inducible regions for C11 were delineated between -243 and -112 and -682 and -427. None were found for B10 which explains its insensitivity to sodium butyrate. However, one butyrate-sensitive area within B10 was defined when placed upstream of the cellular fos promoter. This new inducible region mapped to a distal site between -1049 and -769. The same region found to sensitize C11 in its natural context (-243 to -112) was functional in fos. We have also shown that these B10 and C11 butyrate-sensitive sequences share homologies with HIV-1 3' LTR butyrate-responsive regions. Finally, we have identified a 17 nucleotide sequence common to elements that confer butyrate responsiveness to heterologous genes.

The identification of distinct butyrate-sensitive areas within the 5'-ends of two members of the serine protease family is very interesting. It emphasizes the concept that these two cytotoxic T cell products are regulated in a different manner and their 5'-upstream regions bind different factors. It also confirms the results obtained from earlier studies which indicated that C11 and B10 share minimal sequence homology within their 5'-upstream regions and contain distinct negative and positive regulatory sequences (Lobe et al., 1989, Frégeau and Bleackley, 1991a). The experiments performed in this report and the previous ones (Frégeau and Bleackley, 1991a,b) used the MTL2.8.2 type II cell line which represents a biological system where constitutive regulation of C11 and B10 prevails. Based on the results accumulated so far, it is possible that the proximal regulatory butyrate-sensitive regions of B10 and C11 genes would be implicated in their basal regulation in the MTL2.8.2 cells. On the other hand, the distal regulatory butyrate-responsive sequences could be involved in inducible regulation of C11 and B10 in the type I cytotoxic T cell clones which are both IL2- and antigen-dependent. These studies and those aimed at looking at butyrate sensitivity in this system are in progress.

BIBLIOGRAPHY

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P. and Karin, M. (1987). Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated trans-acting factor. *Cell* **49**:729-739.

Arai, K., Lee, F., Miyajima, A., Miyatake, S., Arai, N. and Yokota, T. (1990). Cytokines: coordinators of immune and inflammatory responses. *Ann. Rev. Biochem.* **59**:783-836.

Berke, G. (1989). The cytolytic T lymphocyte and its mode of action. *Immunol. Lett.* **20**:169-178.

Birnboim, H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**:1513-1523.

Birren, B.W. and Herschman, H.R. (1986). Regulation of the rat metallothionein-I gene by sodium butyrate. *Nucleic Acids Res.* **14**:853-867.

Birren, B.W., Taplitz, S.J. and Herschman, H.R. (1987). Butyrate-induced changes in nuclease sensitivity of chromatin cannot be correlated with transcriptional activation. *Mol. Cell. Biol.* **7**:3863-3870.

Bleackley, R.C., Lobe, C.G., Duggan, B., Ehrman, N., Frégeau, C., Meier, M., Letellier, M., Havele, C., Shaw, J. and Paetkau, V. (1988). The isolation and characterization of a family of serine protease genes expressed in activated cytotoxic T lymphocytes. *Immunol. Rev.* **103**:5-19.

Bleackley, R.C., Paetkau, V. and Havele, C. (1982). Cellular and molecular properties of an antigen-specific cytotoxic T lymphocyte line. *J. Immunol.* **128**:758-767.

Boffa, L.C., Gruss, R.J. and Allfrey, V.G. (1981). Manifold effects of sodium butyrate on nuclear function. *J. Biol. Chem.* **256**:9612-9621.

Bohan, C.A., Robinson, R.A., Luciw, P.A. and Srinivasan, A. (1989). Mutational analysis of sodium butyrate inducible elements in the human immudeficiency virus type I long terminal repeat. *Virology* **172**:573-583.

Bohan, C., York, D. and Srinivasan, A. (1987). Sodium butyrate activates human immunodeficiency virus long terminal repeat-directed expression. *Biochem. Biophys. Res. Comm.* **148**:899-905.

Boral, A.L., Okenquist, S.A. and Lenz, J. (1989). Identification of the SL3-3 virus enhancer core as a T-lymphoma cell-specific element. *J. Virol.* **63**:76-84.

Boyle, W.J., Smeal, T., Defize, L.H.K., Angel, P., Woodgett, J.R., Karin, M. and Hunter, T. (1991). Activation of protein kinase C decreases phosphorylation of c-jun at sites that negatively regulate its DNA-binding activity. *Cell* **64**:573-584.

Chirgwin, J.M., Przybyla, A.E., McDonald, R.J. and Rutter, W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294-5299.

Chou, J.Y., Sartwell, A.D., Lei, K.-J. and Plouzek, C.A. (1990). Effects of sodium butyrate on the synthesis of human pregnancy-specific β1-glycoprotein. *J. Biol. Chem.* **265**:8788-8794.

Dorner, A.J., Wasley, L.C. and Kaufman, R.J. (1989). Increased synthesis of secreted proteins induces expression of glucose-regulated proteins in butyrate-treated Chinese hamster ovary cells. *J. Biol. Chem.* **264**:20602-20607.

Frégeau, C.J. and Bleackley, R.C. (1991a). Transcription of two cytotoxic cell protease genes is under the control of different regulatory elements. (accepted for publication in *Nucleic Acids Research*).

Frégeau, C.J. and Bleackley, R.C. (1991b). Factors influencing transient expression in cytotoxic T cells following DEAE dextran-mediated gene transfer. *Somat. Cell Mol. Genet.* **17**:239-257.

Fujita, T., Shibuya, H., Ohashi, T., Yamanishi, K. and Taniguchi, T. (1986). Regulation of human interleukin-2 gene. *Cell* **46**:401-407.

Ghosh, H. and Baltimore, D. (1990). Activation *in vitro* of NFκB by phosphorylation of its inhibitor IκB. *Nature* **344**:678-682.

Gilman, M.Z., Wilson, R.N. and Weinberg, R.A. (1986). Multiple protein-binding sites in the 5'-flanking region regulate c-fos expression. *Mol. Cell. Biol.* **6**:4305-4316.

Glineur, C., Zenke, M., Beug, H. and Ghysdael, J. (1990). Phosphorylation of the v-erbA protein is required for its function as an oncogene. *Genes Dev.* **4**:1663-1676.

Gonzalez, G.A., Menzel, P., Leonard, J., Fischer, W.H. and Montminy, M.R. (1991). Characterization of motifs which are critical for activity of the cyclic AMP-responsive transcription factor CREB. *Mol. Cell. Biol.* **11**:1306-1312.

Gorman, C.M. and Howard, B.H. (1983). Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. *Nucleic Acids Res.* **11**:7631-7648.

Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell Biol.* **6**:4305-4316.

Graves, B.J., Johnson, P.F. and McKnight, S.L. (1986). Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene. *Cell* **44**:565-576.

Grusby, M.J., Nabair, H., Wong, H., Dick, R.F., Bluestone, J.A., Schotz, M.C. and Glimcher, L. (1990). Cloning of an IL4 inducible gene from cytotoxic T lymphocytes and its identification as a lipase. *Cell* **60**:451-459.

Haddad, P., Jenne, D., Tschopp, J., Clement, M.V., Mathieumahul, D. and Sasportes, M. (1991). Structure and evolutionary origin of the human granzyme-H gene. *Intern. Immunol.* **3**:57-66.

Hirt, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**:365-369.

Imagawa, M., Chiu, R. and Karin, M. (1987). Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. *Cell* **51**:251-260.

Jenne, D., Rey, C., Haefliger, J.-A., Qiao, B.-Y., Groscurth, P. and Tschopp, J. (1988). Identification and sequencing of cDNA clones encoding the granule-associated serine proteases granzymes D, E and F of cytolytic T lymphocytes. *Proc. Natl. Acad. Sci. USA* **85**:4814-4818.

Kruh, J. (1982). Effects of sodium butyrate, a new pharmacological agent, on cells in culture. *Mol. Cell. Biochem.* **42**:65-82.

Lamph, W.W., Dwarki, V.J., Ofir, R., Montminy, M. and Verma, I.M. (1990). Negative and positive regulation by transcription factor cAMP-response element-binding protein is modulated by phosphorylation. *Proc. Natl. Acad. Sci. USA* **87**:4320-4324.

Lazar, M.A. (1990). Sodium butyrate selectively alters thyroid hormone receptor gene expression in GH3 cells. *J. Biol. Chem.* **265**:17474-17477.

Lenardo, M.J. and Baltimore, D. (1989). NF-κB: a pleiotropic mediator of inducible and tissue-specific gene control. *Cell* **58**:227-229.

Lenardo, M., Pierce, J.W. and Baltimore, D. (1987). Protein-binding sites in Ig gene enhancers determine transcriptional activity and inducibility. *Science* **236**:1573-1577.

Levy-Wilson, B. (1981). Enhanced phosphorylation of high-mobility-group proteins in nuclease-sensitive mononucleosomes from butyrate-treated HeLa cells. *Proc. Natl. Acad. Sci. USA* **78**:2189-2193.

Lobe, C.G., Shaw, J., Frégeau, C., Duggan, B., Meier, M., Brewer, A., Upton, C., McFadden, G., Patient, R.K., Paetkau, V.H. and Bleackley, R.C. (1989). Transcriptional regulation of two cytotoxic T lymphocyte-specific serine protease genes. *Nucleic Acids Res.* 17:5765-5779.

Lowrey, D.M., Aebischer, T., Olsen, K., Lichtenheld, M., Rupp, F., Hengartner, H. and Podack, E.R. (1989). Cloning, analysis, and expression of murine perforin 1 cDNA, a component of cytolytic T-cell granules with homology to complement component C9. *Proc. Natl. Acad. Sci. USA* **86**:247-251.

McKnight, S.L. and Kingsbury, R. (1982). Transcriptional control signals of a eukaryotic protein-coding gene. *Science* **217**:316-324.

Mickley, L.A., Bates, S.E., Richert, N.D., Currier, S., Tanaka, S., Foss, F., Rosen, N. and Fojo, A.T. (1989). Modulation of the expression of a multidrug resistance gene (mdr-1/P-glycoprotein) by differentiating agents. *J. Biol. Chem.* **264**:18031-18040.

Naranjo, J.R., Mellström, B., Auwerx, J., Mollinedo, F. and Sassone-Corsi, P. (1990). Unusual c-fos induction upon chromaffin PC12 differentiation by sodium butyrate: loss of fos autoregulatory function. *Nucleic Acids Res.* **18**:3605-3610.

Oliva, R., Bazett-Jones, D.P., Locklear, L. and Dixon, G.H. (1990). Histone hyperacetylation can induce unfolding of the nucleosome core particle. *Nucleic Acids Res.* **18**:2739-2747.

Ostergaard, H.L. and Clark, W.R. (1989). Evidence for multiple lytic pathways used by cytotoxic T lymphocytes. *J. Immunol.* **143**:2120-2126.

Partington, G.A., Yarwood, N.J. and Rutherford, T.R. (1984). Human globin gene transcription in injected *Xenopus* oocytes: enhancement by sodium butyrate. *EMBO J.* **3**:2787-2792.

Perry, C.A. and Annunziato, A.T. (1989). Influence of histone acetylation on the solubility, H1 content and DNaseI sensitivity of newly assembled chromatin. *Nucleic Acids Res.* **17**:4275-4291.

Podack, E.R. (1985). Molecular mechanism of lymphocyte-mediated tumor cell lysis. *Immunol. Today* **6**:21-27.

Randak, C., Brabletz, T., Hergenröther, M., Sobotta, I. and Serfling, E. (1990). Cyclosporin A suppresses the expression of the interleukin 2 gene by inhibiting the binding of lymphocyte-specific factors to the IL-2 enhancer. *EMBO J.* **9**:2529-2536.

Rius, C., Cabanas, C. and Aller, P. (1990). The induction of vimentin gene expression by sodium butyrate in human promonocytic leukemia U937 cells. *Exp. Cell Res.* **188**:129-134.

Schmidt, R.E., MacDermott, R.P., Bartley, G., Bertovich, M., Amato, D.A., Austen, K.F., Schlossman, S.F., Stevens, R.L. and Ritz, J. (1985). Specific release of proteoglycans from human natural killer cells during target lysis. *Nature* **318**:289-291.

Smith, M.R. and Greene, W.C. (1989). The same 50-kDa cellular protein binds to the negative regulatory elements of the interleukin 2 receptor α-chain gene and the human immunodeficiency virus type 1 long terminal repeat. *Proc Natl. Acad. Sci. USA* **86**:8526-8530.

Stoddart, J.H., Lane, M.A. and Niles, R.M. (1989). Sodium butyrate suppresses the transforming activity of an activated N-ras oncogene in human colon carcinoma cells. *Exp. Cell Res.* **184**:16-27.

Tang, D.-C. and Taylor, M.W. (1990). Transcriptional activation of the adenine phosphoribosyltransferase promoter by an upstream butyrate-induced Moloney murine sarcoma virus enhancer-promoter element. *J. Virol.* **64**:2907-2911.

Tang, S.-J., Ko, L.-W., Lee, Y.-H. and Wang, F.-F. (1990). Induction of *fos* and *sis* proto-oncogenes and genes of the extracellular matrix proteins during butyrate induced glioma differentiation. *Biochim. Biophys. Acta* **1048**:59-65

Thiesen, H.-J., Bösze, Z., Henry, L. and Charnay, P. (1988). A DNA element responsible for the different tissue specificities of Friend and Moloney retroviral enhancers. *J. Virol.* **62**:614-618.

Thomas, P.S. (1980). Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA* **77**:5201-5205.

Tschopp, J. and Corzelmann, A. (1986). Proteoglycans in secretory granules of NK cells. *Immunol. Today* **7**:135-136.

Tschopp, J. and Nabholz, M. (1990). Perforin-mediated target cell lysis by cytolytic T lymphocytes. *Ann. Rev. Immunol.* **8**:279-302.

Wirth, T., Staudt, L. and Baltimore, D. (1987). An octamer oligonucleotide upstream of a TATA motif is sufficient for lymphoid-specific promoter activity. *Nature* **329**:174-178.

Young, J.D.-E (1989). Killing of target cells by lymphocytes: a mechanistic view. *Physiol. Rev.* **69**:250-314.

CHAPTER V

GENERAL DISCUSSION AND CONCLUSIONS

Cytotoxic T lymphocytes are members of a network of cells whose role is to protect against invasion. Recent developments in molecular immunology have provided powerful tools for the elucidation of the mechanism by which CTLs mediate the destruction of their targets. As a result, many components of the "killing" process have recently been isolated and characterized. The present investigation examined the signals responsible for the transcriptional activation of two members of the CTL-specific serine protease family, namely C11 (CCP1) and B10 (CCP2), that are believed to play an important role in the process of cell-mediated lysis.

The approach described here has been referred to as the "reverse genetics". It involves two steps: 1) the delineation of the control regions of inducible genes such as the serine proteases and 2) the identification of the distinct proteins that bind each of the defined control regions. These nuclear proteins are presumably targets for signals emanating from the antigen receptor. This strategy links the events that take place on the cell surface with those occurring in the T cell nucleus, and has been used successfully in the study of IL2, IL3, IL2 receptor α chain, GM-CSF and G-CSF gene regulation (Arai et al., 1990, Crabtree, 1989, Ullman et al., 1990). While these analyses have focussed mainly on helper T cells, much remains to be done to define the genetic controls that prevail during activation of CTLs.

Earlier studies have shown that the expression of C11 and B10 is restricted to CTLs and correlates with the acquisition of cytotoxic potential (Lobe et al., 1986). In time course experiments, the level of both serine protease mRNAs increased dramatically when antigen-dependent cytotoxic T cell lines or spleen cell cultures were activated by antigen or Con A (Lobe et al., 1986). Furthermore, the two mRNAs appeared to be sequentially regulated in response to stimulation; C11 mRNA being detected approximately 12 hours before B10. Nuclear run-on transcription experiments revealed that the increase in C11 and B10 mRNA corresponds to transcriptional induction of the two genes (Lobe et al., 1989), thus suggesting that regulation of the C11 and B10 genes takes place at the transcriptional level.

Following these studies, the 5'-upstream regions of both genes were examined for the presence of DNAse1 hypersensitive sites usually associated with transcriptionally active genomic domains. The chromatin in the 5'-ends of C11 and B10 genes was shown to be relatively decondensed implying that these regions of DNA were accessible to transcription factors (Lobe et al., 1989). DNAse1 hypersensitive sites were mapped at positions -1000 and -100 in B10 and -400 and -100 for C11. These analyses led to the present study on the identification of the regulatory elements within the 5'-flanking regions of C11 and B10 and their respective binding factors.

As a first step toward the identification of the regulatory elements of the C11 and B10 genes, the transcription initiation sites for each gene were mapped (Lobe et al., 1989). C11 and B10 promoting regions appear very different from the typical eukaryotic promoter or housekeeping gene promoter. The usual TATAA and CCAAT boxes are not present although C11 has a CATAAA element at position -30 which may function as a TATA box. B10 does not show any sequences that resemble a TATA consensus. C11 contains two GC boxes located respectively between -80 and -57 and -446 and -442, homologous to two of the GC boxes found in the DHFR gene promoter (Blake et al., 1990). These have been shown to play an important role in transcriptional initiation of certain genes. The functional properties of these elements in C11 will come from mutagenesis and deletion experiments. The determination of the minimal sequences required for basal expression of both serine proteases awaits further investigation.

Since the coding regions of the C11 and B10 genes are so similar and since both are induced in response to antigenic stimulation, it was expected that they should share common regulatory elements. Therefore, a search for homology between the C11 and B10

5'-flanking sequences and for the presence of known regulatory elements within these regions was performed. The search revealed the presence of a number of potential regulatory motifs, some of which were shared between the two genes (Tables V.1 and V.2). These common elements included: CLE2 (conserved lymphokine element 2), CRE (cAMP responsive element), AP1 (activation protein 1), NFκB (nuclear factor kappa B), IgH (immunoglobulin heavy chain enhancer core sequences), IL2/NFAT purine box (IL2-specific nuclear factor of activated T cells purine box), TCRα (T cell receptor α enhancer core), c-myc (negative element) and SV40/MoMLV enhancer core motifs. The genetic elements distinct to C11 included CLE1, AP2, OCT (octamer motif), two GC boxes I and II. On the other hand, B10 had sequences homologous to the Tα2 enhancer, IL2Rα (IL2 receptor α negative element) and SV40 enhancer core (reversed orientation). While some of these consensus sequences have been associated specifically with lymphoid genes (CLE1, CLE2, IgH, IL2/NFAT, TCR, Tα2), others have been detected in a wide variety of genomic contexts (CRE, AP1, AP2, NFκB, SV40 enhancer, OCT).

To determine the functional significance of these genetic elements in C11 and B10 gene regulation, a transfection protocol was first developed for the introduction of exogenous DNA into cytotoxic T cells (see Chapter II). A number of transfection methods were tested using the MTL2.8.2 type II cytotoxic T cell line. Electroporation, calcium phosphate coprecipitation, lipofection and polybrene-assisted gene transfer did not provide any encouraging results. Only the DEAE dextran-mediated transfer gave significant and reproducible transfection efficiencies coupled with low toxicity. The DEAE dextran protocol was optimized for the transfection of a transcription reporter construct pRSVcat. In agreement with studies performed by other investigators, CTLs showed a very narrow window of susceptibility to DNA transfections. This may explain why CTLs have been notably difficult to transfect following standard protocols.

A number of parameters were optimized to achieve maximal *cat* expression in the cloned cytotoxic T cell line MTL2.8.2. One of the very interesting aspects of the procedure

Element	Sequence	Position
C11		
CLE2:	TCAAGGTA	(-813 to -806)
NFκB-like:	GAGAATGACTCCC	(-795 to -783)
AP2:	CCCCCACCCC	(-767 to -758), (-203 to -194)
OCT:	GTTTGCAT	(-633 to -626)
NFAT:	AAGAAGTAGGAATG	(-603 to -590)
AP1:	TCAGTCAGT, TGAGTCAT	(-572 to -564), (-150 to -143)
CLE1:	AGTGATTCCAC	(-566 to -556)
GC box I:	GGCTGATGGGTGCTTGGTGGGCC	(-446 to -424)
GRE:	TGTTCT	(-390 to -385)
IgH:	TCACTAGATGGTCA	(-345 to -332)
IgH:	TACAAGAGATGAGC	(-317 to -304)
c-myc:	AGATGAG	(-311 to -305)
NFκB-like:	GGGACTCTGATACC	(-230 to -217)
MMLV:	CTTGTGGTCTT, CTGTGGTTA	(-187 to -177), (-127 to -119)
CLE1:	ATGATTCTCAC	(-114 to -104)
CRE:	AGACGTCA	(-91 to -84)
GC box II:	GGCAGGGGCAGTAGGGCAGGAG	(-80 to -59)
CD3 δ:	AAGCAGA	(-25 to -19)
TATA-like box:	TCATAAAA	(-31 to -24)

TABLE V.1 Homologies between the 5'-flanking sequences of C11 and known regulatory elements. The nucleotide sequences represent the C11 motifs that show greater than 80 % homology with previously described elements. Comparisons were made from sequence data from Arai et al., 1990 (CLE1 and CLE2, NFκB), Lenardo and Baltimore, 1988 (NFκB), Imagawa et al., 1987 (AP2), Wirth et al., 1987 (OCT), Fujita et al., 1986 and Randak et al., 1990 (NFAT-purine box), Angel et al., 1987 (AP1), Blake et al., 1990 (GC boxes), Novak and Rothenberg, 1987 (GRE), Lenardo et al., 1987 (IgH), Boral et al., 1989 and Georgopoulos et al., 1988 (MMLV and SV40 enhancer core), Roesler et al., 1988 and Müller et al., 1989 (CRE), Tunnacliffe et al., 1986 (CD3 δ).

Element	Sequence	Position
B10		
IgH:	ATGTAGAGCAGAGAGC	(-1253 to -1238)
Tα2:	CAGAGAGCCACATAC	(-1245 to -1231)
c-myc:	CCTCTCTT	(-1136 to -1129)
IgH:	ACACACACCTATG	(-1018 to -1006)
CRE:	TGAGGTCAG	(-998 to -990)
IL2Rα:	TCTCACCTCCCAGAG	(-985 to -971)
IgH:	CACTTGGTCATTGGATATGGGGCAA	(-623 to -599)
G-CSF:	ATTCTGCAAT	(-462 to -453)
NFAT:	AAGGAAGGAA	(-368 to -359)
Purine stretch:		(-371 to -275)
AP1:	TGCAGTCA	(-219 to -212)
NFκB-like:	GATGGACTTCCT	(-210 to -199)
SV40 rev.:	ACCACA	(-179 to -174)
IL2α:	TTCATCCCAG	(-146 to -137)
CLE2:	TCAGGAGGTCA	(-125 to -115)
SV40 or MMLV:	TGTGGTAACAGTGTC	(-112 to -98)
CRE:	TGTGACGTCA	(-63 to -54)
CD3δ:	AAGCAGA	(-48 to -42)

TABLE V.2 Homologies between the 5'-flanking sequences of B10 and known regulatory elements. The nucleotide sequences represent the B10 motifs that show greater than 80 % homology with previously described elements. Comparisons were made from sequence data from Lenardo et al., 1987 (IgH enhancer core), Ho et al., 1990 (Tα2), Roesler et al., 1988 and Müller et al., 1989 (CRE), Smith and Greene, 1989 (IL2 receptor α chain element), Ullman et al., 1990 and Arai et al., 1990 (G-CSF), Karim et al., 1990 and Ullman et al., 1990 (NFAT-purine box), Angel et al., 1987 (AP1), Lenardo and Baltimore, 1989 and Arai et al., 1990 (NFκB), Jones et al., 1988 (SV40 enhancer core reversed), Arai et al., 1990 (CLE2), Jones et al., 1988 and Boral et al., 1989 (SV40 and MMLV enhancer core), Tunnacliffe et al., 1986 (CD3δ).

was the short adsorption time required to obtain optimal *cat* gene expression. Only 15 minutes at room temperature were sufficient to reach a plateau in *cat* activity; even a 5 minute-adsorption period gave a reasonable level of enzyme activity. This represents the shortest time employed to transfect cells and obtain high efficiency as well as good cell viability. It was also interesting to observe that none of the usual "facilitators" used to improve transfection efficiencies or transcription of the reporter genes were functional in the MTL2.8.2 cell line. Treatments with DMSO, glycerol, PEG or chloroquine diphosphate had no effect. However, a 12 to 13 hour exposure to sodium butyrate at a concentration of 7.5 mM at 37°C increased the level of *cat* activity by a factor of five in the case of pRSVcat.

When tested with the C11 or B10 5'-flanking region/cat fusion constructs, this adapted protocol worked well and a significant level of *cat* gene expression was detected. This transfection method provided an excellent means to start delineating the differences between two of the CTL-specific serine protease 5'-flanking regions. Although the protocol was not rigorously optimized for other T cell lines, it was shown to be appropriate for type I cytotoxic T cells which depend on both IL2 and antigen for their growth (see Appendix to Chapter II). These cells represent a more relevant biological system for the study of immune mechanisms but the difficulty in obtaining large numbers of cells for experimental purposes has prevented their frequent use. Type I cells were successfully transfected using the 5 minute-adsorption period procedure as evidenced by the level of RSV-directed *cat* gene expression obtained with the quiescent cells. Following stimulation with PMA/dibutyryl cAMP/IL2 or antigen/IL2, this level increased 3 to 4 fold. Thus type I cells are also amenable to transfection by this adapted DEAE dextran method.

The mapping of the regulatory elements of C11 and B10 was achieved through the generation of sequential 5'-deletion fragments fused to the reporter *cat* gene which were assayed for their ability to direct the transcription of the *cat* gene following transfection into a variety of cell types (see Chapter III). Both C11 (896 bp) and B10 (1080 bp) full length

5'-flanking regions directed *cat* gene expression specifically in cytotoxic T cells suggesting the presence of CTL-specific sequences. No expression was detected in the helper T cell lines EL4 and D10 or in the fibroblast L cells. The various 5'-deletion constructs further delineated positive and negative areas that mapped to different locations within the 5'-upstream regions of C11 and B10. When ligated to heterologous promoters such as the basal cellular fos and the strong viral herpes simplex thymidine kinase (tk), the modulating regions lost their CTL-specificity; they worked in both the CTL and helper T cell lines as well as in fibroblasts. This suggested that CTL specificity is achieved through the combined action of multiple regulatory DNA elements and their trans-interacting factors. This combinatorial aspect of gene expression was further noted when the functional properties of the positive sequences were studied in the context of ubiquitously expressed promoters. Some of the positive elements of C11 and B10 delineated in the natural 5'-context lost their stimulatory effect when placed in other genomic contexts such as the c-fos or viral tk. Thus, the function of the C11 and B10 regulatory elements appears to be dictated not only by the factors that bind to them but also by the neighboring sequences which are target sites for other binding factors that participate in the formation of transcriptional complexes . This is akin to the situation described for numerous eukaryotic genes (see section C in Introduction).

Two positive regulatory sequences were mapped for C11 (-682 to -427 and -243 to +68) and one for B10 (-279 to -189). In addition, each flanking region contains a region of DNA (B10 -1617 to -1049 and C11 -427 to -243) that has a negative influence on transcription. The position of C11 and B10 regulatory sequences coincide with DNAse1 hypersensitive sites defined in a previous study (Lobe et al., 1989). These sites were detected in CTL clones but not in unstimulated thymocytes. They correspond to chromatin domains that have an altered structural conformation which results in higher susceptibility to pancreatic DNAse1 digestion. They are usually associated with transcriptionally active or competent regions of genes most probably because trans-acting factors within these regions disrupt the packaging of the DNA into nucleosomes. Thus, the regulatory sequences within C11 and B10 5'-ends presumably activate or repress transcription by binding nuclear proteins. Preliminary footprint experiments indicate that some areas in the 5'-upstream regions of C11 and B10 are indeed protected from DNAse1 digestion by proteins in fractionated nuclear extracts from MTL2.8.2 cytotoxic T cells (Figure V.1). So far, the sites that were found within the protected areas include, for C11: the CRE, MoMLV enhancer core (two sites), AP1, IgH E1 enhancer core, IL2/NFAT-purine box and two novel motifs referred to as X and Y. For B10: the proximal CRE, SV40/MMLV enhancer core, CLE2, SV40 enhancer core (reversed), NFκB-like motif, IL2/NFAT-purine box, IgH enhancer core, two novel motifs referred to as W and Z and the purine stretch were protected from DNAse1 digestion. From these footprints, only a few were found within the regulatory regions mapped in C11 and B10: the Z motif, AP1 and NFκB-like sequence located between -279 and -189 (+ region in B10), the NFAT-purine box, X and Y elements between -682 and -427 (+ region in C11), the MMLV/SV40 enhancer core sequences and AP1 site between -243 and -112 (+ region in C11). However, the presence of footprints does not necessarily mean that the bound factors are able to mediate transcription; they may require post-transcriptional modifications such as phosphorylation to become active. This has been seen for CREB where a conformational change induced by phosphorylation is required for its activation (Gonzalez et al., 1991). In addition, the presence of footprints over ubiquitous binding sequence motifs does not exclude the possibility that these are target sites for cognate factors that are involved in the formation of higher order transcription complexes which are distinct for each gene. It is also possible that different factors share the same ubiquitous binding motif.

The topographical maps, although preliminary, suggest that C11 and B10 bind common as well as distinct factors at different positions within their 5'-flanking regions. This further implies that these two genes, while sharing significant homology within their transcribed regions and being clustered together on chromosome 14, may be targets for

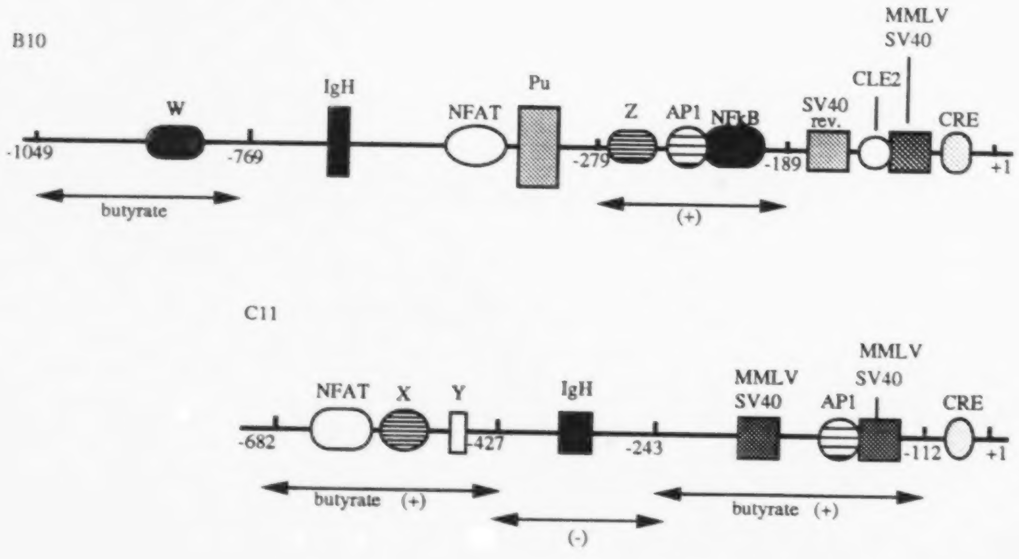


FIGURE V.1 Diagram of the preliminary DNase1 footprints in the B10 and C11 5'-flanking regions. Nuclear extracts from MTL2.8.2 cells were prepared as described by Ohlsson and Edlund (1986). HindIII/EcoRI fragments representing different regions of the 5'-flanking sequences of B10 and C11 were labelled at the HindIII end with [^{32}P]αCTP. DNase1 reactions were done in a final volume of 50 μl containing 25 mM Hepes pH 7.8, 50 mM KCl, 0.05 mM EDTA, 0.5 mM DTT. 0.5 mM PMSF, 5% glycerol. Reactions contained 1-2 ng of end-labelled fragment and 1 μg of poly dI.dC. 50-100 μg of nuclear extract was incubated on ice with poly dI.dC for 20 min., after which the DNA was added and the reaction allowed to continue 10-15 min. at room temperature. $MgCl_2$ and $CaCl_2$ concentrations were adjusted to 5 mM and 1 mM respectively and 500 ng of DNase1 added; the reactions were set for 45 sec. Control DNA was digested with 5 ng of DNase1 for the same amount of time. All reactions were stopped by the addition of 100 mM Tris-HCl pH 8.0, 100 mM NaCl, 1% sarkosyl, 10 mM EDTA, 100-300 μg/ml of proteinase K and 25 μg/ml of calf thymus DNA; incubated at 37°C for 15 min. then at 95°C for 2 min. These mixtures were then phenol-chloroform (1:1) extracted and the DNA fragments ethanol precipitated. The resultant fragments were analysed on 6% polyacrylamide gels containing 7 M urea. CRE: cAMP responsive element, SV40 or MMLV: SV40 or Moloney nurine leukemic virus enhancer core, AP1: activating protein 1, IgH: immunoglobulin heavy chain enhancer core, X, Y, W, Z: novel motifs not previously described, NFAT: IL2-specific NFAT-purine box, NFκB: NFκB-like motifs.

distinct transcriptional complexes responsible for their CTL-specific and sequential expression. The data accumulated so far show that C11 and B10 share certain genetic elements with genes that are expressed during the maturation of T cells in the thymus. These include the AP1 and the CRE binding sites. Thus, the transcriptional regulation of these genes may share common features.

As discussed in section A of the Introduction, two signals are required to activate a precursor CTL: antigen and IL2. Triggering the T cell receptor with antigen activates protein kinase C and increases the intracellular calcium level. Tyrosine phosphorylation signals are also transduced from the lymphoid cell tyrosine kinase associated with the CD8 receptor. These initial signals cause a transient burst of transcription of a few genes, including the transferrin receptor, the IL2 receptor α subunit and presumably some transcriptional factors involved in the activation of these proteins. The increase in C11 and B10 mRNA appears to occur later, after this initial response (see Table I.4, Introduction). Their expression is most likely contingent on the IL2 interaction with its cognate receptor which produces a rapid redistribution of kinase C from the cytosol to the plasma membrane where it is activated. It induces the production of secondary messengers which differ from the classical DAG and IP3. As well, the tyrosine kinase associated with the IL2 receptor phosphorylates cytoplasmic proteins involved in the activation of genes. It is reasonable to suspect that C11 and B10 regulatory sequences might respond to signals emanating from the IL2 receptor since their expression depends on the presence of this cytokine.

Interestingly, both C11 and B10 have NFκB-like motifs within their 5'-flanking regions. It is well documented that this transcriptional factor becomes activated only after its release from a complex with the IκB protein (Lenardo and Baltimore, 1989, Urban and Baeuerle, 1990). The release is induced by the phosphorylation of the IκB factor. In CTLs, signals originating from the IL2 receptor (IL2 kinase) may activate NFκB or other factors that bind NFκB-like motifs and favour their transit into the nucleus where they bind their cognate sequences and turn on the C11 and B10 serine protease expression. This would be similar to the situation seen for another member of the serine protease multigene family, serine protease B, which is involved in the complement cascade alternative pathway and is transcriptionally activated by IL1. It has been shown that this activation is mediated by a nuclear factor having a binding specificity identical to that of NFκB (Nonaka and Huang, 1990). It is possible, therefore, that NFκB or a closely related factor may mediate the IL2 signal in CTL cells that leads to the acquisition of cytotoxic potential. These authors have also indicated that the NFκB-like element present in the serine protease B 5'-flanking region can act as an IL1-responsive element, but only in the context of its surrounding sequences. A similar element in the C4 serine protease gene was nonresponsive. Thus, coordination between trans-acting factors is required for induction of genes in general. In this perspective, it is possible that the C11 NFκB-like element may be functional while the B10 NFκB-like motif may not. The converse may also apply. Another possibility might be that the transcription of several "late" proteins including C11, which may take place prior to B10 transcription, depletes the available pool of NFκB. In this instance, the assumption is made that both B10 and C11 NFκB-like motifs bind the same transcriptional factor. A recent report by Hohmann et al., (1991) indicated that after exhaustion of the preexisting NFκB factors from NFκB/IκB complexes, *de novo* protein synthesis is required to replenish the pool of active NFκB. A lag period would therefore be registered between the C11 transcription and B10.

The AP1 binding motif (which has been shown to respond to agents that activate protein kinase C) is found in both the C11 and the B10 5'-upstream regions. This genetic element has been detected in "early" mitogen-response genes and appears to be involved in the early regulatory events of T cell activation. The AP1 site in C11 corresponds to a true AP1 motif with its sequence TGAGTCAT (-150 to -143), however, the motif in B10 is different from the consensus sequence, TGCAGTCA (-219 to -212). It is possible, therefore, that the AP1 site in B10 is not functional. The mere presence of an AP1 site in C11 (true footprint) may be responsible for the fact that C11 transcription always precedes that of B10. If activation of the factors that bind to the AP1 motif requires kinase C or the involvement of the IL2 kinase, the formation of transcriptional complexes might be induced which would result in the activation of C11.

The presence of the CRE binding motif and the fact that it corresponds to a protected region in the 5'-ends of C11 and B10 also suggest that these genes respond to agents that elevate the intracellular level of cAMP. Furthermore, gel mobility shift and competition assays revealed that the C11 CRE sequence competes very effectively for the c-fos CRE motif which has been shown to act as a target site for factors involved in the fos cAMP-regulated gene expression (Grant McFadden, personal communication). Thus, the C11 CRE element may represent a *bone fide* binding site for factors implicated in the cAMP-regulated serine protease gene activation. A third result, which emphasizes the importance of this CRE sequence in the transcriptional activation of C11 and B10, was obtained from Northern blots prepared with CTL mRNA following treatment with dibutyryl cAMP or forskolin. Both agents were found to influence the steady-state level of the serine protease mRNA (Cheryl Helgason, personal communication). cAMP is known to affect the transcription of several eukaryotic genes by activating a cytoplasmic or nuclear cAMP-dependent protein kinase (protein kinase A). Phosphorylation would provoke conformational changes in binding factors and, in turn, promote their participation in complexes responsible for the transcriptional activation of the serine protease genes.

Stimulation of CTLs with antigen and IL2 is a requisite for the transcription of C11 and B10 genes. However, the effect of developmental signals that are transmitted through this effector pathway can be mimicked by agonists of protein kinases such as PMA (protein kinase C) and cAMP (protein kinase A). When type I cells were transfected with some of the constructs containing the positive regulatory elements from C11 and B10 5'-flanking regions and were treated with PMA and dibutyryl cAMP, the levels of *cat* activity detected in these cells were consistently 2 to 3 fold higher than those measured in the quiescent type I cells. PMA or dibutyryl cAMP alone did not stimulate expression of the *cat* gene directed by the C11 or B10 5'-upstream sequences. These experiments suggested that both PMA and dibutyryl cAMP are required for the activation of *cat* expression and both agents act synergistically to activate this expression in type I cells. These results are in agreement with a recent report by Hanson and Ley (1990) who showed that PMA and dibutyryl cAMP regulate the transcription of the human homologue of C11 in PEER cells. Thus, it appears that distinct signal transduction pathways converge to regulate C11 and B10 expression and multiple steps may be involved in the activation process. PMA, dibutyryl cAMP and IL2 may separately direct distinct events that are necessary but not sufficient for the serine protease gene expression in CTLs.

Transcriptional activation of a wide variety of genes by cAMP, PMA, TPA have been reported to proceed via a κB-like binding motif. At least three different proteins, NFκB, H2TF1 and PRDII-BF1 can recognize the κB-like binding motif. This provides evidence for the ubiquitous role of NFκB in transducing extracellular signals. AP1 is also at the receiving end of a complex pathway responsible for transmitting the effects of phorbol esters from the plasma membrane to the transcriptional apparatus. Both sites are present in C11 or B10 and correspond to footprints. They are likely involved in the control of expression of these two genes. Signals initiated through two cell membrane receptors can be integrated at the level of the responsive genes. Two separate studies have recently shown that transcriptional activation of the IL2 and c-fos genes via different effector pathways or stimuli require distinct upstream regulatory sequences (Fraser et al., 1991, Sheng et al., 1988). Similarly, activation of the serine protease genes may involved the participation of different binding factors in the formation of distinct complexes depending on the original stimuli. DNAse1 footprinting experiments performed on quiescent type I cells and activated T cells following different stimulation should determine if different regulatory sequences are used as target sites for binding factors.

During optimization of the DEAE dextran procedure, the level of *cat* activity directed by the Rous sarcoma virus LTR was shown to be significantly increased following exposure to sodium butyrate for 13 hours. This stimulatory effect was also observed when the C11 5'-upstream region was used to drive the *cat* transcription. However, no enhanced effect was noted for B10 5'-flank. Sodium butyrate appeared to have a differential effect on the serine proteases 5'-flanking sequences (Chapter II). Further experiments performed with sodium butyrate were instrumental in the study of C11 and B10 gene regulation (Chapter IV). The transfected C11/*cat* fusion construct as well as the endogenous C11 gene examined in MTL2.8.2 cells were particularly sensitive to butyrate. Both followed the same profile of modulation after exposure to butyrate for 13 hours. B10, however, did not show any modulation of transcription following butyrate treatment at the transfected as well as the endogenous levels. Further examination of the 5'-flanking sequences of C11 and B10 revealed the presence of butyrate-sensitive areas which correspond to some of the proximal and distal regulatory regions defined earlier. Surprisingly, the B10 5'-flank which was not responsive to butyrate also contain one area which by itself is sensitive to the agent. These results could be explained following the series of experiments performed with type I cells.

Antigen-stimulated type I cells treated with butyrate for 13 hours showed a pattern of endogenous and transfected C11 gene expression opposite to the one of type II cells. Instead of an increase in transcription, a down-regulation was noted (Cheryl Helgason, personal communication) and see Table V.3. A similar situation was observed for B10. Experiments with a few of the 5'-deletion fusion constructs indicated that the elements that were defined as butyrate-sensitive in MTL2.8.2 cells corresponded to antigen-responsive elements in type I cells. This suggests that the down-regulation observed may result from the interference of butyrate with the signals that are transduced from the cell surface antigen receptor. The same sequences appear to be targets for both butyrate action and signals emanating from the triggered T cell receptor. Based on this series of experiments, a model is presented in Figure V.2 where the proximal regions are important in the "basal" and CTL-specific expression of C11 while the distal regions are particularly involved in the

Constructs	(%) Chloramphenicol Acetylation Quiescent	Activated (-) butyrate	Activated (+) butyrate
pB10 1080Fcat	2.5	3.8	2.1
pB10 310Fcat	3.2	6.1	3.5
pC11 896Fcat	1.8	3.5	1.9
pC11 439Fcat	2.8	7.2	3.2

TABLE V.3 Effect of sodium butyrate on the levels of *cat* activity driven by the c-fos promoter in the presence of C11 and B10 5'-sequences in antigen/IL2-stimulated type I cells. 2 x 10^7 cells were taken at day 3 following antigen stimulation (see Appendix to Chapter II for stimulation protocol) and were transfected with 15 µg of each construct and 500 µg of DEAE dextran in 1 ml of transfection buffer for 5 min. at room temperature. Cells were washed and placed in complete medium RHFM supplemented with IL2 (30 units/ml) and sodium butyrate (7.5 mM) for 13 hours at 37°C in a 5% CO_2 atmosphere. They were then fed fresh medium depleted of the drug and were incubated for 35 hours before *cat* determination. Levels of *cat* activity shown represent absolute values.

inducible expression of the serine protease genes. Further experiments to examine this hypothesis are in progress.

I would like to point out, however, that the conclusions drawn from this study rely primarily on *cat* assays. Transient gene expression assays have limitations and care must be used both in the design of experiments and in the interpretation of the data. The major cause of assay variability is differences in transfection efficiency. To correct for those variations, several investigators have used cotransfected reporter genes as controls (i.e. β-galactosidase or luciferase). Such corrective measures aimed at normalizing the levels of *cat* activity are not without their limitations (Hollon and Yoshimura, 1989). For example, the use of a control reporter gene must take into consideration competition between promoters of the cointroduced plasmids for limiting amounts of trans-acting transcription factors, some of which may be shared by the respective promoters. The studies reported herein have corrected for transfection-induced variation in assay data by measuring the amount of reporter plasmid DNA in the nuclei of transfected cells, thus avoiding concerns about transcription factor competition.

Another potential drawback of the transient transfection technique is the possibility of competition between the endogenous and newly introduced sequences for common transcription factors. This phenomenon, referred to as "squelching", may result in biased interpretation of the data (Ptashne and Gann, 1990). This problem can be overcome by using retroviral vectors which allow stable expression and low copy integration of the gene of interest into the host chromosome.

Many analyses aimed at improving the understanding of the regulation of the serine protease genes should be performed. Competition studies with oligonucleotides from the protected regions will indicate if separate factors with distinct characteristics bind these sites or if cooperativity between factors is a prerequisite for binding. As well, deletion of specific sites should provide clues on the nature of the complexes that are required for C11 and B10 transcription. Site-specific mutagenesis experiments will help define the motifs required for the binding of specific factors responsible for the CTL-specific expression of the serine proteases. Reconstitution experiments whereby the transcription factors are isolated through affinity columns and allowed to bind to a fragment of DNA to reconstitute the sequence of events that lead to the formation of complexes and activation of gene expression promise to be interesting. Such experiments should determine precisely the reasons behind the sequential expression of C11 and B10. In addition, it will be interesting to compare these protected regions obtained using the type II MTL2.8.2 cells with those of non-CTL lines such as EL4 (helper T) or L (fibroblasts) to define the ubiquitous sequences or those sequences that are not CTL-specific. Gel retardation assays in the presence of

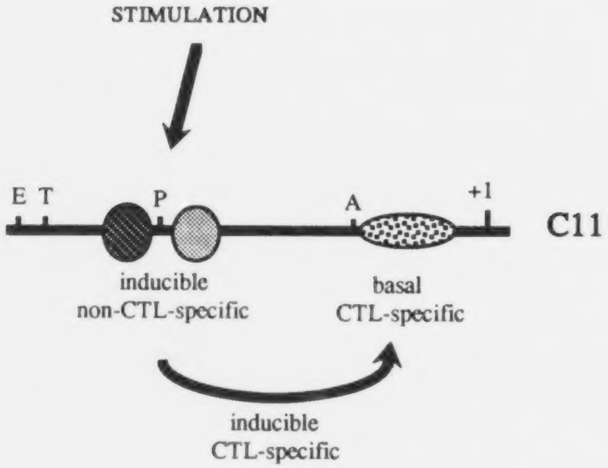

STIMULATION
E
T
P
A
+1
C11
inducible
non-CTL-specific
basal
CTL-specific
inducible
CTL-specific

FIGURE V.2 Model of transcriptional activation for the C11 gene. In the MTL2.8.2 cells, the proximal regulatory elements are responsible for the "basal" and CTL-specific expression of C11. Upon stimulation of the cells with antigen and IL2, the distal elements participate in the formation of transcriptional complexes which together with the proximal region induce the specific expression of the C11 gene in CTL lines.

phosphatases or kinases will determine the requirement for dephosphorylation or phosphorylation of factors prior to transcriptional complex formation.

The aim of the present work was a comparison of the transcriptional regulation of two members of the serine protease gene family. The results obtained provide compelling evidence for the combinatorial and distinct nature of the serine protease C11 and B10 gene regulation. Two positive regulatory regions (-682 to -427 and -243 to +68) and a negative modulatory area (-427 to -243) were mapped within the 5'-end of C11. The B10 5'-flanking region also contains sequences that exert a positive effect (-279 to -189) and negative effect (-1617 to -1049) on transcription. The positive regions are located within DNAse1 hypersensitive sites and confer CTL-specificity to C11 and B10 expression in their natural context. In addition, this cell-specific expression is achieved through the combined action of factors that bind to various target sequences in the 5'-upstream regions of the genes. Both C11 and B10 genes share cis-acting elements such as the CRE, AP1, NFκB, IgH enhancer core, MMLV/SV40 enhancer core and also have distinct motifs including OCT, AP2 (for C11) and Tα2 (for B10) which might play important roles in their respective transcriptional regulation. These elements map to very different locations within the 5-'end of the genes. Butyrate-sensitive sequences were also detected within the 5'-flanking regions of C11 and B10 (C11, -682 to -427 and -243 to -112; B10, -1049 to -769). These regions correspond to some of the regulatory areas previously defined and support the notion that they represent recognition sequences for transcriptional factors. Preliminary DNase1 footprinting analysis suggests that the CRE, AP1 and NFκB motifs are implicated in the regulation of the serine protease gene expression. The regulation of C11 and B10 appears to depend on both antigen/IL2 or PMA/dibutyryl cAMP/IL2 which indicates that signals initiated through different cell membrane receptors may be integrated at the level of the responsive gene. All these experiments indicate that the two CTL-specific genes are regulated differently and are targets for distinct transcriptional complexes. It will be interesting to examine those members of the serine protease family that are more related to B10 and see if they also share similar transcriptional controls. While many key experiments remain to be done to elucidate the precise transcriptional events behind the sequential expression of C11 and B10, the experimental approach developed here has opened the way and provided a few important signposts to guide future investigators. This information can now be used to modulate the expression of the serine proteases at will and better define their role in cell-mediated cytolysis.

" The man who removed the mountain began by carrying away small stones."

_ Chinese Proverb

BIBLIOGRAPHY

Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P. and Karin, M. (1987). Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. *Cell* **49**:729-739.

Arai, K., Lee, F., Miyajima, A., Miyatake, S., Arai, N. and Yokota, T. (1990). Cytokines: coordinators of immune and inflammatory responses. *Ann. Rev. Biochem.* **59**:783-836.

Blake, M.C., Jambou, R.C., Swick, A.G., Kahn, J.W. and Azizkhan, J.C. (1990). Transcriptional initiation is controlled by upstream GC-box interactions in a TATAA-less promoter. *Mol. Cell. Biol.* **10**:6632-6641.

Boral, A.L., Okenquist, S.A. and Lenz, J. (1989). Identification of the SL3-3 virus enhancer core as a T-lymphoma cell-specific element. *J. Virol.* **63**:76-84.

Crabtree, G.R. (1989). Contingent genetic regulatory events in T lymphocyte activation. *Science* **243**:355-361.

Fraser, J.D., Irving, B.A., Crabtree, G.R. and Weiss, A. (1991). Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. *Science* **251**:313-316.

Fujita, T., Shibuya, H., Ohashi, T., Yamanishi, K. and Taniguchi, T. (1986). Regulation of human interleukin-2 gene: functional DNA sequences in the 5' flanking region for the gene expression in activated T lymphocytes. *Cell* **46**:401-407.

Georgopoulos, K., van den Elsen, P., Bier, E., Maxam, A. and Terhorst, C. (1988). A T cell-specific enhancer is located in a DNAse1-hypersensitive area at the 3' end of the CD3 δ gene. *EMBO J.* **7**:2401-2407.

Gonzalez, G.A., Menzel, P., Leonard, J., Fischer, W.H. and Montminy, M.R. (1991). Characterization of motifs which are critical for activity of the cyclic AMP-responsive transcription factor CREB. *Mol. Cell. Biol.* **11**:1306-1312.

Hanson, R.D. and Ley, T.J. (1990). Transcriptional activation of the human cytotoxic serine protease gene CSP-B in T lymphocytes. *Mol. Cell. Biol.* **10**:5655-5662.

Ho, I.-C., Bhat, N.K., Gottschalk, L.R., Lindsten, T., Thompson, C.B., Papas, T.S. and Leiden, J.M. (1990). Sequence-specific binding of human Ets-1 to the T cell receptor α gene enhancer. *Science* **250**:814-818.

Hohmann, H.-P., Rémy, R., Scheidereit, C. and van Loon, A.P.G.M. (1991). Maintenance of NF-κB activity is dependent on protein synthesis and the continuous presence of external stimuli. *Mol. Cell. Biol.* **11**:259-266.

Hollon, T. and Yoshimura, F.K. (1989). Variation in enzymatic transient gene expression assays. *Anal. Biochem.* **182**:411-418.

Imagawa, M., Chiu, R. and Karin, M. (1987). Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. *Cell* **51**:251-260.

Jones, N.C., Rigby, P.W. and Ziff, E.B. (1988). *Trans*-acting protein factors and the regulation of eukaryotic transcription: lessons from studies on DNA tumor viruses. *Genes Dev.* **2**:267-281.

Karim, F.D., Urness, L.D., Thummel, C.S., Klemsz, M.J., McKercher, S.R., Celada, A., Van Beveren, C., Maki, R.A., Gunther, C.V., Nye, J.A. and Graves, B.J. (1990). The ETS-domain: a new DNA-binding motif that recognizes a purine-rich core DNA sequence. *Genes Dev.* **4**:1451-1453.

Lenardo, M.J. and Baltimore, D. (1989). NF-κB: a pleiotropic mediator of inducible and tissue-specific gene control. *Cell* **58**:227-229.

Lenardo, M., Pierce, J.W. and Baltimore, D. (1987). Protein-binding sites in Ig gene enhancers determine transcriptional activity and inducibility. *Science* **236**:1573-1577.

Lobe, C.G., Finlay, B.B., Paranchych, W., Paetkau, V. and Bleackley, R.C. (1986a). Novel serine proteases encoded by two cytotoxic T lymphocyte-specific genes. *Science* **232**:858-861.

Lobe, C.G., Havele, C. and Bleackley, R.C. (1986b). Cloning of two genes that are specifically expressed in activated cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* **83**:1448-1452.

Lobe, C.G., Shaw, J., Frégeau, C., Duggan, B., Meier, M., Brewer, A., Upton, C., McFadden, G., Patient, R.K., Paetkau, V.H. and Bleackley, R.C. (1989). Transcriptional regulation of two cytotoxic T lymphocyte-specific serine protease genes. *Nucleic Acids Res.* **17**:5765-5779.

Müller, U., Roberts, M.P., Engel, D.A., Doerfler, W. and Shenk, T. (1989). Induction of transcription factor AP-1 by adenovirus E1A protein and cAMP. *Genes Dev.* **3**:1991-2002.

Nonaka, M. and Huang, Z.-M. (1990). Interleukin-1-mediated enhancement of mouse factor B gene expression via NFκB-like hepatoma nuclear factor. *Mol. Cell. Biol.* **10**:6283-6289.

Novak, T.J., White, P.M. and Rothenberg E.V. (1990). Regulatory anatomy of the murine interleukin-2 gene. *Nucleic Acids Res.* **18**:4523-4533.

Ohlsson, H. and Edlund, T. (1986). Sequence-specific interactions of nuclear factors with the insulin gene enhancer. *Cell* **45**:35-44.

Ptashne, M. and Gann, A.A.F. (1990). Activators and targets. *Nature* **346**:329-331.

Randak, C., Brabletz, T., Hergenröther, M., Sobotta, I. and Serfling, E. (1990). Cyclosporin A suppresses the expression of the interleukin-2 gene by inhibiting the binding of lymphocyte-specific factors to the IL-2 enhancer. *EMBO J.* **9**:2529-2536.

Roesler, W.J., Vandenbark, G.R. and Hanson, R.W. (1988). Cyclic AMP and the induction of eukaryotic gene transcription. *J. Biol. Chem.* **263**:9063-9066.

Sheng, M., Dougan, S.T., McFadden, G. and Greenberg, M.E. (1988). Calcium and growth factor pathways of *c-fos* transcriptional activation require distinct upstream regulatory sequences. *Mol. Cell. Biol.* **8**:2787-2796.

Smith, M.R. and Greene, W.C. (1989). The same 50-kDa cellular protein binds to the negative regulatory elements of the interleukin 2 receptor α-chain gene and the human immunodeficiency virus type 1 long terminal repeat. *Proc. Natl. Acad. Sci. USA* **86**:8526-8530.

Tunnacliffe, A., Sims, J.E. and Rabbitts, T.H. (1986). T3δ pre-mRNA is transcribed from a non-TATA promoter and is alternatively spliced in human T cells. *EMBO J.* **5**:1245-1252.

Ullman, K.S., Northrop, J.P., Verweij, C.L. and Crabtree, G.R. (1990). Transmission of signals from the T lymphocyte antigen receptor to the genes responsible for cell proliferation and immune function: the missing link. *Ann. Rev. Immunol.* **8**:421-452.

Urban, M.B. and Baeuerle, P.A. (1990). The 65-kD subunit of NF-κB is a receptor for IκB and a modulator of DNA-binding specificity. *Genes Dev.* **4**:1975-1984.

Wirth, T., Staudt, L. and Baltimore, D. (1987). An octamer oligonucleotide upstream of a TATA motif is sufficient for lymphoid-specific promoter activity. *Nature* **329**:174-178.